JOURNAL OF AGRICULTURAL RESEARCH

VOL. 41 WASHINGTON, D. C., OCTOBER 1, 1930 No. 7

STUDIES ON INFECTIOUS HAIRY ROOT OF NURSERY APPLE TREES[1]

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INTRODUCTION

The enlargements that commonly occur on underground parts of piece-root grafted nursery apple trees have received attention from numerous investigators.

Smith and his coworkers (*25*),[3] in their extensive researches on crown gall, established *Bacterium tumefaciens* Smith and Town.[4] as the organism whose several varying strains produce different types of crown gall on many plants, including apple. After giving careful consideration to the question of whether they should include in one rather variable species all the strains of crown-gall bacteria studied or should split them into different species, these workers (*25, p. 157–158*) concluded that—

> * * * much further time will be required to decide positively whether it is best to regard all crown galls as due to variations of one polymorphous species, or whether they should be separated into two or more species * * * and for the present at least we prefer to leave the group undivided, merely indicating the various cultures for purposes of convenience by the name of the plant from which derived, as daisy, peach, poplar, etc.

After the work of Smith and his associates a widespread tendency developed to attribute to the crown-gall organism various apple overgrowths whose cause had not been clearly shown. Later investigations, however, initiated by Smith (*24*), have tended to modify this conception. Lek (*9*) has given a review of the literature on various overgrowths.

Studies on control reflect the complexity of the problem and suggest that several factors influence the development of overgrowths. Riker, Keitt, and Banfield (*21*) effected marked reduction in the number of malformed trees by wrapping well-fitted grafts with adhesive tape.

[1] Received for publication May 1, 1930; issued October, 1930. Published with the approval of the director of the Wisconsin Agricultural Experiment Station. These studies were begun as a part of the Wisconsin program in the crown-gall project supported cooperatively by the Crop Protection Institute, the Iowa State College of Agriculture and Mechanic Arts, and the University of Wisconsin, have been continued in cooperation with the U. S. Department of Agriculture. This work has been supported in part by a grant from the special research fund of the University of Wisconsin.

[2] Died May 3, 1929.

[3] Reference is made by number (italic) to Literature Cited, p. 538.

[4] Synonym: *Phytomonas tumefaciens* (Smith and Town.) Committee S. A. B. This name is used in the present paper.

Melhus, Muncie, and Fisk (*13*) reported increasing the percentage of smooth trees by the use of wedge grafts. Riker (*18*) suggested the influence of incompatibility between scion and stock for consideration in relation to some of the enlargements encountered. Wormald and Grubb (*30*) reported the reduction of knots following chemical and mechanical treatments. On the other hand, Waite and Siegler (*29*) reported increasing the percentage of smooth trees by the use of cloth and raffia wrappers with treatments in hydroxymercurichlorophenol. They attribute the reduction in overgrowths to the action of the chemical on the crown-gall bacteria.

Siegler (*23*) isolated cultures of bacteria from malformations on nursery apple trees and conducted inoculation tests with them. He states (*23, p. 312*): "Just how important a rôle this apple strain of *Bacterium tumefaciens*, if in fact it is a strain of that organism, plays in the formation of the woolly-knot type of malformation is still undetermined."

The present writers (*20, 22, 35, 36*) have made preliminary reports on the isolation of an organism which is different from *Phytomonas tumefaciens* and which causes hairy root. A more detailed report of their studies [5] on hairy root and its causal organism is the purpose of the present paper.[6]

DESCRIPTION OF INFECTIOUS HAIRY ROOT

The infectious hairy root under consideration is most commonly observed on grafted nursery apple trees 1, 2, or 3 years old as they are dug for storage in the fall. The nurserymen have applied the names "hairy root" and "woolly knot" to these malformations because of the very large number of small roots protruding either directly from the stems or roots or from localized hard swellings that frequently occur at the unions. (Fig. 1.) In the very early stages of the development of these malformations the "hairy" roots are fleshy; in intermediate stages mixtures of the fleshy and fibrous types are found; and in the later stages the fibrous type predominates. Since it has been shown by Harris (*4*) that moisture is an important factor in determining whether a root will be fleshy or fibrous, additional work needs to be done to ascertain how much the hairy root organism influences the type of roots produced. The enlargement often found at the base of the roots in the later stages is usually covered by a definite cortex, which, however, may be obscure because of the profusion of small roots extending through it. The interior of the basal enlargement is hard, being composed largely of woody elements, which occur in an irregular cross-grain position and supply vascular connection with the roots. In many cases, especially between the roots, small pockets are found which inclose particles of soil and a large number of soil fungi and bacteria. Typical examples of hairy root that followed inoculations may be seen in Figure 2.

In addition to the hairy-root enlargements just described, there are similar malformations which resemble the knots from which the hairy roots emerge, except that the roots have not developed. Such knots have a typical structure characterized by a very deeply convoluted surface from which fissures may extend through a tortuous

[5] The experimental work was done at Madison, Wis., unless otherwise stated.

[6] Since this manuscript was prepared, additional papers dealing with this subject have appeared (*2a, 6a, 14a, 17a, 25a*).

path for some distance into the enlargement. These fissures often contain soil particles and a variety of soil microorganisms. Although

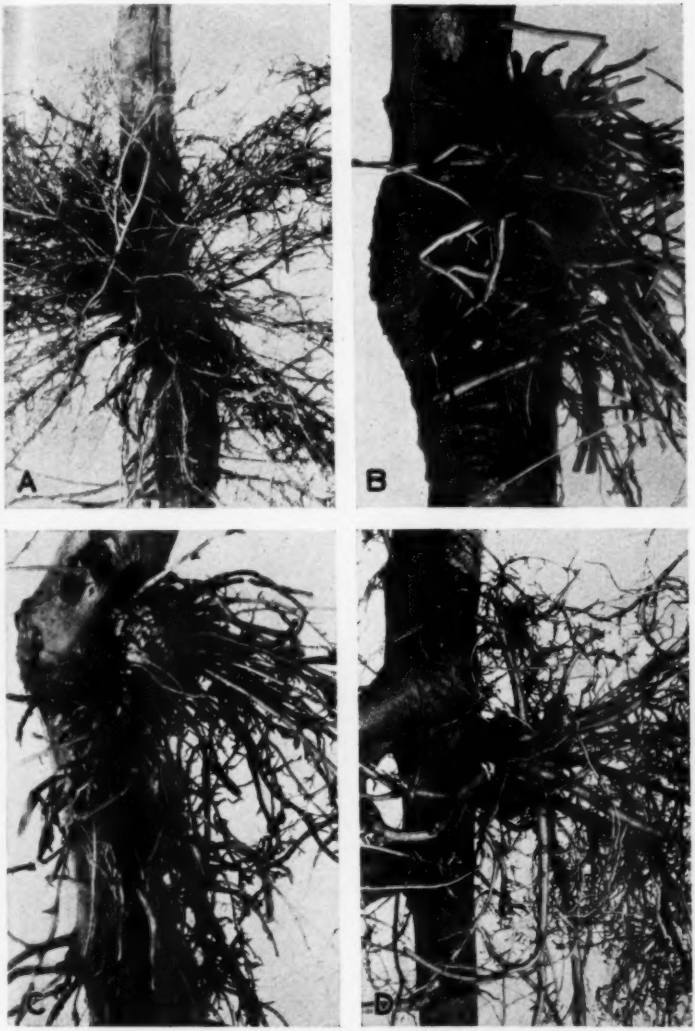

FIGURE 1.—Natural infection of hairy root on 3-year-old Wealthy apple trees from the nursery row. A and B.—Hairy-root development from little or no basal enlargement, showing predominance of fibrous roots in A and of fleshy roots in B. C and D.—Hairy-root development of the "woolly knot" type from comparatively small overgrowths

the etiology is still uncertain, typical hairy-root organisms have been obtained from this kind of knot and will be described later. A typical example is shown in Figure 3, D. Riker and Keitt (*19*) were uncertain

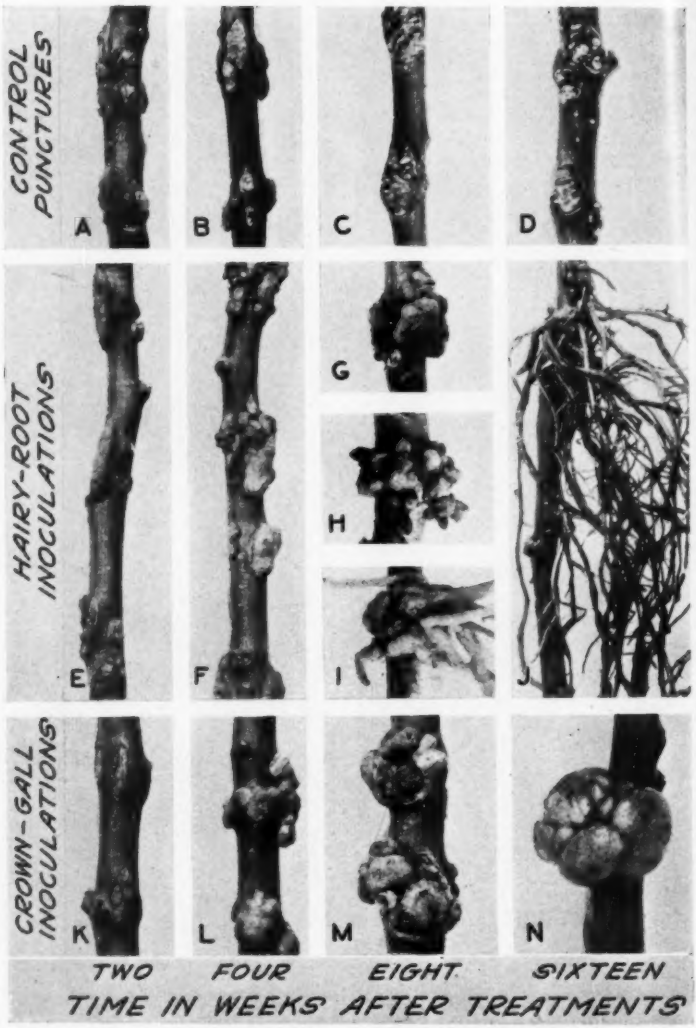

FIGURE 2.—Different stages in the development of enlargements on Wealthy apple scion wood below ground following certain treatments. A, B, C, and D.—Responses to uninoculated needle punctures at approximately 2, 4, 8, and 16 weeks after treatment. E, F, G–H–I, and J.—Responses to inoculations with the hairy-root organism at the same intervals of time. G, H, and I.—Different examples of the responses to this organism 8 weeks after inoculation. At the stage shown in G and earlier stages the response to the hairy-root organism might easily be confused with that to the crown-gall organism. At the stage shown in H the roots are beginning to appear, while in I they may have become somewhat elongated. K, L, M, and N.—Responses to inoculations with the crown-gall organism at the same intervals of time as the other series

of the etiology of overgrowths of the two types just described, which yielded bacteria of doubtful identity. A continuation of the study

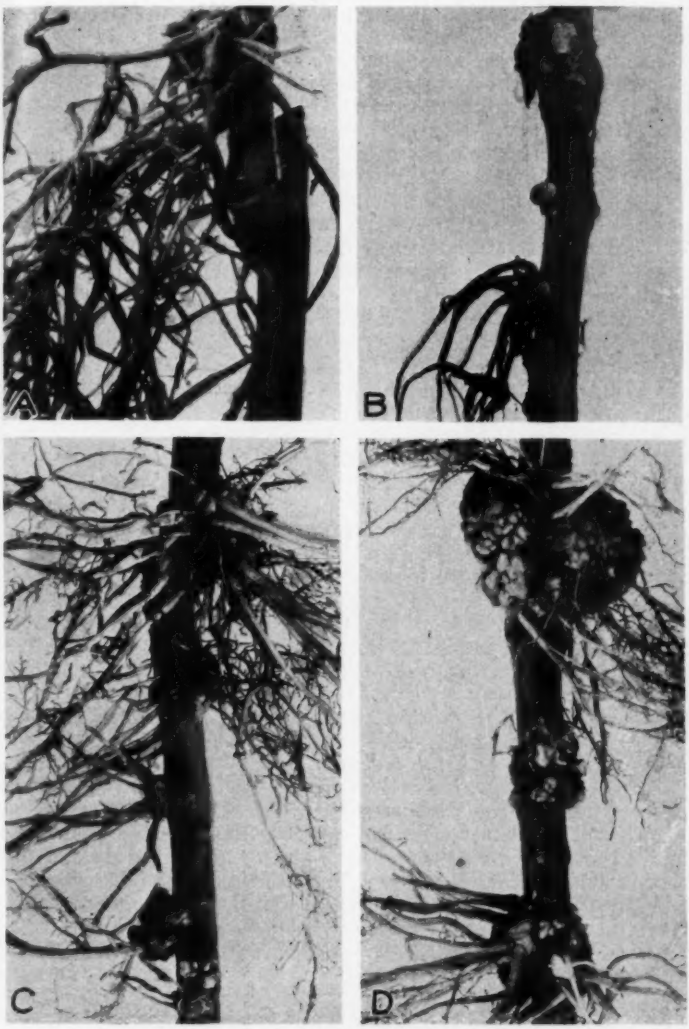

FIGURE 3.—Successive stages in the response of Wealthy apple grafts to puncture inoculations made with the hairy-root organism in the spring of 1927. A.—Dug in the fall of 1927. B.—Dug in the spring of 1928; shows immature roots which were winter killed and early stages of new hairy-root development. C.—Dug in the fall of 1928. D.—Dug at the same time as C; shows deeply convoluted type of overgrowth often found on nursery trees in their second year. Further work will be necessary to show conclusively whether or not this is an uncomplicated form of hairy root

of these cultures of unknown identity led to the investigation reported in the present paper.

Mixtures of the characters of the malformations just mentioned with those of overgrowths due to other causes are also found.

The very early stages of infectious hairy root on underground parts of nursery apple trees closely resemble those of crown gall and excess callus. Studies on the differentiation of crown-gall and hairy-root enlargements in these early stages of development on apple are not yet finished. In these stages it seems at present unwise to attempt such a distinction except as a result of bacteriological analysis. However, after a sufficient incubation period, the swelling induced by *Phytomonas tumefaciens* becomes larger and shows no tendency to develop root primordia or roots. That induced by the hairy-root organism typically soon develops root primordia and roots. Illustrations of these types of malformation in different stages are shown in Figure 2. The characters of hairy root, crown gall, and wound tissue in their early stages are discussed further in the section on seasonal-development studies.

In inoculation experiments with the hairy-root organism on apple stems aboveground, malformations which resemble small burrknots have been induced. Siegler (*23*) reports similar results from inoculations with the cultures which he isolated from overgrowths on apples. In the present writers' experiments, although root primordia were sometimes produced, no roots were developed in the air except under artificial conditions of high humidity.

The resemblances to burrknots thus shown by malformations induced by inoculations with the hairy-root organism in the open air might raise a question (*23*) concerning the validity of the work of Brown (*2*), Riker and Keitt (*19*), and Hatton, Wormald, and Witt (*5*). The present writers, using Patel's method (*16*), have attempted isolations from 14 typical burrknots of various ages on apple received in 1928 from West Virginia through the courtesy of N. J. Giddings and his staff. In each case, in accordance with the work just cited (*23*), negative results were obtained. The majority of the isolation plates were completely sterile, and in no case was any colony secured which closely resembled the crown-gall or hairy-root organisms. The evidence now available seems to be against the conclusion that most of the common, naturally occurring burrknots are of parasitic origin. When burrknots occur beneath the surface of the ground and roots are produced from them, as for example on Paradise and Doucin stocks, the resulting formation is very similar to hairy root. As far as the present writers are aware a clear differentiation between these underground burrknots and hairy root on the basis of external characters has not yet been worked out.

Examinations of the hairy root of apple seedlings, which was studied by Muncie (*14*), have not been included in the work reported in the present paper.

ISOLATION AND INOCULATION STUDIES

Isolation and inoculation experiments with the hairy-root organism were begun in connection with the studies of crown gall and wound overgrowth of apple nursery stock reported by Riker and Keitt (*19*). By using a carefully standardized technic they found that *Phytomonas tumefaciens* was readily isolated from a type of malformation which they considered to be the true bacterial crown gall and that the organism isolated induced typical crown gall when inoculated into

susceptible plants, such as tomato, geranium, tobacco, and apple. However, when the same technic was applied to apple malformations of various other types, very different results were obtained. In some cases the isolation plates either remained sterile or developed colonies of bacteria which induced no overgrowth when inoculated into tomato, geranium, tobacco, and apple. In other cases organisms were isolated which closely resembled *P. tumefaciens* in colony characters, but which induced only doubtful or slight overgrowth responses in inoculation tests. In all cases these inoculations were made on tomato, and in many instances on apple, tobacco, and geranium. In his criticism of this work because of the host plants employed, Siegler (*23*) apparently failed to notice (1) that, in addition to tomato, geranium, and tobacco, apple was also employed as a test plant for the organisms and (2) that organisms secured from malformations described as typical crown gall on apple were pathogenic on tomato, geranium, and tobacco, as well as on apple. Concerning the identity and possible relation to overgrowths on apple of those bacteria which resembled the crown-gall organism in colony characters but occasioned little or no overgrowth on tomato, conclusions were withheld, pending the results of the further studies which are reported in the present paper.

Further evidence concerning the nature of these bacteria was secured in 1927 by the present writers, using the cultures isolated by Riker and Keitt (*19*). Each of eight selected strains was inoculated by needle punctures into underground parts of the stems of first-season Wealthy apple trees. Six inoculations with each strain were made on each of 10 trees. Fifty trees were similarly punctured for controls but not inoculated. At the time of inoculation the experimental trees had made about 12 inches of new growth. In each case, after the treatments were completed, soil was heaped up over the places treated so as to provide as nearly natural conditions as possible for the incubation of the organism. After two months either hairy root or crown gall showed at the places of inoculation. When the trees were dug in the fall, six of the eight cultures had induced hairy root and two had induced crown gall at the places where the bacteria were inserted. The 50 control trees all gave negative results. The details of these studies are presented in Table 1. Attempts to recover each of the six strains of bacteria which stimulated hairy root from these artificially infected trees yielded bacteria which appeared on agar plates to be typical of those inserted. Cultures recovered from five of these overgrowths were reinoculated into apple in 1928. Each produced overgrowths typical of those from which the isolations were made.

These preliminary experiments made it seem desirable to undertake a detailed study of infectious hairy root. Trees bearing hairy root were secured from nurseries in different localities where it was prevalent and brought into the laboratory for examination.

The procedure in the inoculation work on infectious hairy root during the winter of 1927–28 was similar to that employed by Riker and Keitt (*19*). However, instead of nutrient dextrose agar or yeast-infusion agar, the medium devised by Patel (*16*) was used. This medium contains, per liter: Agar, 17 gm.; bile salt (sodium taurocholate), 3 gm.; glucose, 2 gm.; and crystal violet, 0.002 gm. It is adapted from the

well-known media employed in isolations of organisms of the colon-typhoid group.

TABLE 1.—*Results of a series of puncture inoculations with cultures of the hairy-root and crown-gall organisms in 1927* [a]

Culture (organism and No.)	1-year-old wood			New wood		
	Number of inoculations	Result of inoculations		Number of inoculations	Result of inoculations	
		Hairy root	Crown gall		Hairy root	Crown gall
Hairy root:						
1207	39	12	0	20	15	0
2004	41	10	0	19	12	0
2014	34	5	0	25	21	0
2098	41	11	0	19	10	0
2104	47	12	0	14	7	0
2105	34	2	0	30	12	0
Crown gall:						
2018	47	0	3	11	0	9
2124	47	0	3	11	0	9
Controls	128	0	0	112	0	0

[a] On July 10 each culture was inoculated through needle punctures into both the 1-year-old scions and the new shoots of 10 first-year Wealthy apple trees in the field. Fifty control trees were punctured but not inoculated. All places of inoculation and all control punctures were promptly covered with soil. The results were taken Oct. 28.

The value of this bile medium was compared with that of yeast-infusion agar. The bile agar materially reduced the amount of growth of certain fungi and of some types of bacteria which often occur in the soil and within portions of the hairy-root overgrowths. It seemed to have no inhibitory effect upon the early development of *Phytomonas tumefaciens*, the hairy-root organism, *Bacillus radiobacter* Beij. and Van Deld., or certain nonpathogenic mucoid organisms of the soil. However, these organisms were apt to die on the bile medium after some days. Consequently yeast-infusion agar was used to carry the stock cultures after they were transferred from the isolation plates. The efficiency of Patel's method (*16*), in which bile agar was used, was compared upon 20 different enlargements with that of the method employed by Riker and Keitt (*19*), in which yeast-infusion agar was used. The bile-agar method yielded positive results in 19 cases, while the yeast-infusion agar method gave positive results from all the specimens. This difference was considered to be within experimental error. Although Patel's method was found to be no more accurate in the hands of the present writers than the yeast-infusion agar method, it was used for routine work because of other advantages. Time was saved because only three isolation attempts, instead of five, were made for each enlargement, and difficulty with contaminating organisms was reduced.

During the winter of 1927–28 isolation studies were made upon 88 additional enlargements, chiefly of the hairy-root type and including "woolly knot," which were secured from 12 representative nurseries in Iowa, Kansas, Minnesota, Missouri, Nebraska, Oklahoma, and Wisconsin. Typical specimens are shown in Figure 1. After the isolation plates were poured and individual colonies had developed, certain typical colonies were selected and transfers made for stock cultures.

Each strain of bacteria taken from the isolation plates was later inoculated into apple and tomato plants. In some cases additional inoculations were made on tobacco. The apple was used because it is the host plant with which this investigation is most concerned and is susceptible to both crown gall and hairy root. The tomato was selected as a promising differential host for the crown-gall and hairy-root organisms. In order to minimize the possibility of natural infection, the apple trees were grown under the following conditions: (1) In the open field in ground where apples had not been grown before, (2) in field plots that had been steamed for 25 minutes and boarded up according to the method (7) commonly used for tobacco seed beds, (3) in virgin soil in pots in the greenhouse, and (4) in autoclaved soil in the greenhouse under conditions designed to exclude the chance intervention of any infectious organism. The data are reported without further reference to the conditions under which the apple plants were grown, as like results were obtained by each method used. The earlier inoculations on apple were made in stems of the preceding year's growth. After it was found that the use of stems of the current year's growth reduced the incubation period and greatly increased the chances of obtaining positive results, the inoculations were made in young shoots from first-year grafts. The tomato and tobacco plants were grown in pots in the greenhouse. The bacteria from agar slants were inoculated through needle punctures into the stems of the experimental plants, aboveground on tomato and tobacco and underground on apple. Five punctures were made in each inoculated and in each control plant. Two plants of the apple were inoculated with each bacterial strain tested, and one control plant was punctured but not inoculated. For inoculations into tomato and tobacco, one plant was used for each bacterial strain tested, and one control was left for every five plants inoculated. For purposes of comparison many parallel inoculations were made with carefully purified strains of *Phytomonas tumefaciens* that had been isolated from typical crown galls on apple and other plants.

The results of these isolation and inoculation studies are too voluminous for detailed publication. Records relating to each of the 88 malformations studied were kept under 92 headings. Inoculation tests were made with 359 strains of bacteria, an average of over 4 strains for each overgrowth. Two of the 88 enlargements yielded the crown-gall organism, as determined by inoculations on tomato and apple. Thirteen yielded no organisms that induced response following inoculation. One yielded an organism that occasioned host responses intermediate between those of hairy root and crown gall. The other 72 specimens all yielded organisms that induced reactions characteristic of the hairy-root organism following puncture inoculations on tomato (fig. 4, B) and apple stems (fig. 4, A). Control punctures were all negative. The reactions of the inoculated and control plants are discussed more fully in the section on seasonal development studies and are illustrated in Figure 2. The percentage of cultures from individual enlargements which gave positive results for hairy root varied with the different knots. Some of the overgrowths yielded organisms all of which were able to stimulate hairy root, some yielded only nonpathogenic cultures, and others yielded both kinds. Some of the results are summarized in Table 2, where the specimens

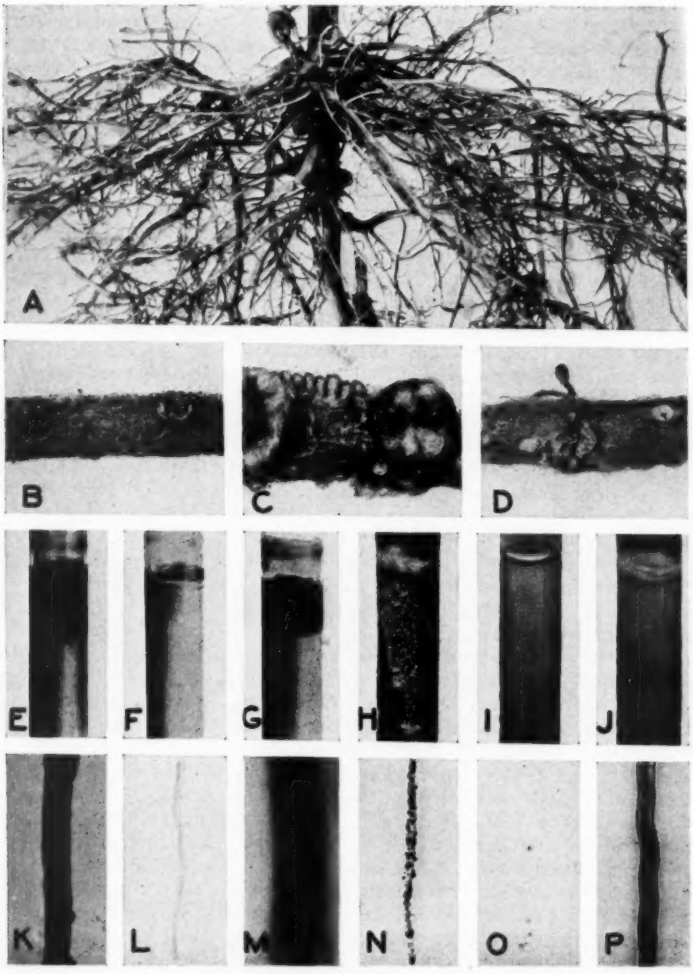

FIGURE 4.—Some results of inoculation and cultural studies of the hairy-root and crown-gall organisms and *Bacillus radiobacter*. A.—Wealthy apple graft four months after puncture inoculation with the hairy-root organism. B, C, and D.—Tomato stems one month after inoculation with the hairy-root organism, the crown-gall organism, and *B. radiobacter*, respectively. E, F, and G.—Growth characters of the crown-gall organism, the hairy-root organism, and *B. radiobacter*, respectively, in litmus milk after three weeks at 28° C. H, I, and J.—Growth characters of the crown-gall organism, the hairy-root organism, and *B. radiobacter*, respectively, in xylose peptone-salt liquid medium after four weeks at 21°. K, L, and M.—Growth characters of the crown-gall organism, the hairy-root organism, and *B. radiobacter*, respectively, in Petri dishes on glycerophosphate mannitol solid medium after two weeks at 21°. N, O, and P.—Growth characters of the crown-gall organism, the hairy-root organism, and *B. radiobacter*, respectively, in Petri dishes on glycerophosphate glucose solid medium containing brom-thymol blue, after two weeks at 21°. In N the bacterial growth was pale blue; in P, dark blue. There was little growth in O

are classified according to the percentage of the strains of bacteria derived from them which gave positive results in inoculation tests.

In the work which has just been reported the hairy-root organism was isolated from a much higher percentage of the specimens cultured than in the earlier work reported by Riker and Keitt (*19*). In this connection the following facts are worthy of note: (1) As their investigation was not planned to include a study of hairy root, comparatively few specimens which showed excessive root development were included in the material which they studied, whereas in the present work on hairy root most of the specimens chosen for the isolation work showed excessive root development; (2) the use of improved methods of grafting, since the earlier work was done, has materially reduced the percentage of root-grafted trees which develop overgrowths, with corresponding changes in the percentages of these overgrowths in relation to cause; and (3) the inoculation technic used in the work reported in the present paper is more efficient in identifying the hairy-root organism than that used by Riker and Keitt.

TABLE 2.—*Results of certain studies of the etiology of hairy root and "woolly knot" on apple, with the specimens classified according to percentage of strains of bacteria derived from them which gave positive results in inoculation tests, 1927–28* [a]

Classes of specimens [b]	Specimens cultured	Average cultures per specimen	Cultures studied	Reactions of cultures on tomato and apple	
				Hairy root	Crown gall
A (100 per cent)	43	3.8	162	157	5
B (50–100 per cent)	18	5.0	90	59	2
C (15–50 per cent)	14	4.1	57	14	0
D (0 per cent)	8	5.6	45	0	0
E (0 per cent)	5	1.0	5	0	0

[a] The sources of the material studied and the methods used in isolating the cultures and in making the inoculations are recorded in the text. These determinations are based upon the results taken from 1,148 inoculations on tomato and 3,104 inoculations on apple. The details of these studies are omitted because of their large volume. These cultures were secured by making transfers in the usual manner from well-isolated colonies in isolation plates, and received no further purification.

[b] The classes are based on the percentage of strains isolated from each specimen which induced the reactions typical of either the crown-gall or the hairy-root organism when inoculated into tomato and apple. The numbers in parentheses show the range of the percentages included in each class.

The parallel inoculations with *Phytomonas tumefaciens* consistently yielded typical crown gall on apple, tomato, and tobacco. None of these plants developed hairy-root symptoms.

The etiology of hairy root has been further studied by means of reisolation and reinoculation experiments. Reisolation from hairy root induced by inoculation has been attempted from 40 specimens and has been successful in each case. The organisms secured from these isolations appeared typical of what was inserted. Thirty-eight representative cultures secured in this work from 12 experimentally produced hairy roots were reinoculated into tomato and apple. Cultures from each of these 12 specimens gave positive results following inoculations on apple.

Inoculation studies were also carried out with a series of crown-gall, hairy-root, and *Bacillus radiobacter* cultures that had been especially purified as reported later in this paper (p. 522). A number of different strains were inoculated by needle punctures into stems of tobacco and tomato. In the field they were inoculated just above the ground by needle punctures into the current season's growth of Wealthy apple grafts. These grafts had been planted in soil that had been steamed by the method described earlier. Immediately after

treatment the places of inoculation were covered to a depth of several inches with steamed soil. A summary of the results of these studies, together with the origin of the cultures used, is given in Table 3, which is discussed later (p. 534).

The limited data now available suggest that the hairy-root organism has a rather wide host range. Positive results have been obtained thus far from inoculations on the following plants, in addition to those already mentioned: Rose (*Rosa setigera* Michx.), honeysuckle (*Lonicera morrowi* Gray), sugar beet (*Beta vulgaris* L., var. *crassa* Alef.), bean (*Phaseolus vulgaris* L.), and Paris daisy (*Chrysanthemum frutescens* L.). Further studies of host range are in progress.

TABLE 3.—*Results of puncture inoculations with certain replated cultures of the hairy-root and crown-gall organisms and Bacillus radiobacter on tomato, tobacco, and apple* [a]

Culture		Tomato and tobacco			Apple		
Organism and source	No.	Number of inoculations	Result of inoculations		Number of inoculations	Result of inoculations	
			Hairy root	Crown gall		Hairy root	Crown gall
Crown gall:							
Rose	T-1	18	0	[b] 5	20	0	0
Almond	T-2	27	4	[b] 14	30	[c] 5	0
Incense cedar	T-3	27	0	23	70	0	0
Apple	T-4	18	0	18	15	0	0
Raspberry	T-5	45	0	45	60	0	7
Do	T-6	41	0	38	50	[c] 2	24
Willow	T-7	23	0	23	10	0	1
Peach	T-11	8	0	8	10	0	0
Raspberry	T-13	37	0	34	10	0	10
Apple	T-16				10	0	0
Peach	T-17	28	0	28	10	0	2
Raspberry	T-30	22	0	32	31	0	22
Do	T-33	15	0	[b] 0	30	0	0
Apple	T-35	29	[d] 0	29	45	[d] 0	10
Peach	T-36	28	0	28	10	0	2
Walnut	T-37	28	0	28	65	[c] 2	10
Apple	T-39	18	0	18	10	0	0
Do	T-40	28	0	28	20	0	12
Hairy root, apple	T-31	8	0	0	20	0	0
Do	T-32	35	18	0	70	18	0
Do	T-34	24	6	0	50	1	0
Do	T-38	28	0	[d] 28	70	33	[d] 0
Do	T-41	19	9	0	55	43	0
Do	T-42	52	22	0	56	38	0
Do	T-43	21	13	0	65	42	0
Do	T-45	27	17	0	40	30	0
Do	T-46	28	13	0	45	36	0
Do	T-47	14	9	0	20	8	0
Do	T-48	23	4	0	20	13	0
Do	T-49	15	10	0	35	22	0
Do	T-50	23	18	0	40	30	0
B. radiobacter, soil	R-1	4	0	0	20	0	0
Do	R-3	4	0	0	30	0	0
Do	R-7	4	0	0	10	0	0
Do	R-9	4	0	0	10	0	0
Do	R-11	4	0	0	25	0	0
Do	R-16	4	0	0	20	0	0
Do	R-20	4	0	0	10	0	0
Do	R-27	4	0	0	10	0	0
Do	R-29	4	0	0	10	0	0
Do	R-31	4	0	0	10	0	0
Controls, punctures only		180	0	0	270	0	0

[a] Several subcultures were obtained from each original culture after the four successive replatings described in the text. The results from the subcultures are summarized for each original culture and reported opposite its number. T-5 was purified by the single-cell technic, the results shown being summarized for five strains.

[b] It appears that the original cultures, which were positive for crown gall on tomato, were mixtures of the crown gall and some nonpathogenic organisms. In the process of purification the pathogenic organism was recovered in some instances, and the nonpathogenic bacteria in others.

[c] In each of these cases one of the three (T-2) or four (T-6 and T-37) purified subcultures produced hairy root. It appears that the original cultures were mixtures of the hairy-root and crown-gall organisms.

[d] Subcultures T-35-1A and T-38-1A appear still to be mixtures of the crown-gall and hairy-root organisms. The overgrowths that followed the inoculations were intermediate in characters between those typical of crown gall and hairy root. They are arbitrarily classified according to the characters which predominated.

SEASONAL DEVELOPMENT

Preliminary studies have been made on the seasonal development of hairy root following puncture inoculations, in comparison with the development of crown gall and the reaction to control punctures. During the spring of 1928, ground that had been used for truck crops in which crown gall is seldom if ever found in nature was selected for experimental plantings. This ground was prepared in the usual manner and then steamed according to the method in common use for tobacco beds (7). The steamed beds were then surrounded by 10-inch boards on edge to prevent the washing of surface soil. While these precautions did not exclude the possibility of the presence of the crown-gall and similar organisms in the experimental ground, it limited their access as much as might reasonably be expected under field conditions. In this steamed soil Wealthy apple grafts were planted as subjects for the study of the development of hairy root and crown gall following inoculation. Approximately 200 plants were inoculated with each organism by needle punctures through masses of the causal bacteria on the underground parts of the scion. When the current year's growth was tall enough inoculations were made also just above the ground on this new tissue. In most cases the needle was passed entirely through the stem. Soil was promptly heaped about the places of inoculation so that they were kept moist and under conditions favorable for root development. Four hundred control plants received the same treatment, except that no bacteria were applied. Attention such as spraying, cultivating, and irrigating was supplied as required throughout the growing season. The progress of the reaction to the different treatments was observed approximately twice a month.

Two weeks after treatment only a very slight difference was apparent between the reactions induced by the inoculations with the crown-gall and hairy-root organisms and those occasioned by the control punctures. In all cases slight enlargements had developed, which on the control plants appeared to be merely a callus response to the needle injury and which on the inoculated plants seemed at this stage to be little more than such a response.

A month after treatment the difference between the reactions of the inoculated plants and the controls was more marked. On the controls the enlargements were very little larger than at the end of the 2-week period, while those of both the hairy-root and crown-gall inoculations were almost twice as large as when last noted. The surface character of both of the latter at this time was of the crown-gall rather than the callus type. (Fig. 2, B, F, and L.)

Two months after inoculation a distinct differentiation in the responses to the three different treatments was evident. The control punctures had corked off in the usual manner, leaving a comparatively small development of wound tissue. (Fig. 2, C.) The crown-gall malformations had continued to enlarge, with the development of typical characters of crown gall. (Fig. 2, M.) No roots were observed to have developed from the gall proper. In exceptional cases in other experiments roots have been observed to push out from bud regions near the crown galls. Such root developments were also rarely observed near the control punctures. The hairy-root overgrowths at this time were in some cases merely enlargements of previous stages (fig. 2, G), while in others they showed the development of root

primordia and of roots (fig. 2, H and I). This root development occurred earlier upon the stems of the current year's growth than on older wood. It is important to note that up to and including this time the results of inoculations with the hairy-root organism on apple might readily be confused with those of crown gall.

Three months after inoculation the effects of the treatments were quite conspicuously differentiable. The control punctures showed no change. The crown galls were larger but showed no change in external characters. From the hairy-root inoculations, however, the roots had grown out in considerable numbers. Many of the early roots were fleshy. A mixture of fleshy and fibrous roots was quite common in the cases of hairy root studied.

Four months after the beginning of the experiment the overgrowths which resulted from the inoculations were observed to have enlarged without further changes in characters. (Fig. 2, D, J, and N.) Subsequent examinations showed additional growth of the hairy-root and crown-gall enlargements until very late in the fall. The white roots characteristic of new growth were apparent long after the top was frostbitten and at the time when the ground was beginning to freeze. These young roots were found to be very susceptible to winter injury. Harris (*4*) reports that young growing roots of apple are commonly very susceptible to injury from low temperatures.

The development of overgrowths following inoculations with the hairy-root organism over a 2-year period has been traced in ordinary field soil. (Table 4.) A considerable number of the trees used for the inoculations of 1927 (Table 1) were held over and grown a second year. In the fall of 1927 the places of inoculation showed abundant hairy-root development as previously described. (Fig. 3, A.) The winter of 1927–28 provided little or no covering of snow over the experimental planting at critical low-temperature periods, and the roots from hairy-root inoculations were killed. (Fig. 3, B.) However, they grew out again very early in the season of 1928, and by fall had matured into well-branched fibrous roots. (Fig. 3, C.) In addition to the second-year root development, peculiarly convoluted enlargements also appeared at many of the places of inoculation. (Fig. 3, C and D.) These were comparatively hard and contained deep fissures in which soil particles were frequently found to be incorporated. The convolutions often appeared superficially to be composed of a mass of rootlike tissue which seemed to grow together into a knot instead of growing out into roots. The histology of this structure has not been worked out. Macroscopically it is quite different from the typical soft gall which follows inoculations with *Phytomonas tumefaciens*. While these developments have appeared with some regularity at the places of inoculation in the second year, more evidence is necessary before they can be attributed without reservation to the hairy-root organism alone. They have appeared in a few cases subsequent to inoculations of the hairy-root organism into underground parts of slow-growing apple stems. From the overgrowths thus produced, bacteria were isolated which appeared to be typical of those inserted and which induced typical hairy root when reinoculated into underground parts of succulent apple stems.

TABLE 4.—*A comparative record over a 2-year period of the development of hairy root and crown gall from inoculations on Wealthy apple nursery trees*

Culture used	Trees treated	Inoculations [a]	Date observed	Overgrowths present			
				Hairy root	Crown gall	Doubtful	None
Hairy root, No. 2104	10	54	Oct. 28, 1927	19	0	14	21
			May 5, 1928	24	0	9	21
			Oct. 31, 1928	30	0	0	24
Crown gall, No. 2018	10	54	Oct. 28, 1927	0	9	7	38
			May 5, 1928	0	10	6	38
			Oct. 31, 1928	0	22	0	[b] 23
Controls	5	30	Oct. 28, 1927	0	0	0	30
			May 5, 1928	0	0	0	30
			Oct. 31, 1928	0	0	0	[b] 24

[a] Hairy-root and crown-gall bacteria, respectively, were inoculated July 7, 1927, by groups of needle punctures into the underground stem parts of first-year apple grafts.
[b] One tree died during the course of the second season of growth.

BACTERIOLOGICAL STUDIES [7]

Since the crown-gall and hairy-root organisms showed such striking differences in the inoculation studies, it seemed desirable to subject them to a comparative bacteriological study. Accordingly, 18 of the cultures isolated in 1926 from apple and other hosts secured from a wide geographic range were run through the common bacteriological examinations. The results of this work showed that the cultures under consideration were somewhat variable in their characters and failed to show a clear differentiation between the crown-gall and hairy-root bacteria. This was entirely in accord with the conclusion reached by Smith et al. (*25, p. 158*).

Consideration of the data secured from the cultures used in 1926 suggested that either (1) the crown-gall species is highly variable or (2) the cultures employed were not pure. The colony characters of these cultures gave no suggestion of contamination. However, it was noticed that, on the media then employed, the colony characters of (1) the crown-gall organism, (2) the organism which was later found to cause hairy root, (3) *Bacillus radiobacter* (*1, 11, 12*), and (4) certain mucoid organisms common in the soil were indistinguishable. Furthermore, it has been commonly found by various workers, following Smith et al. (*25*), that the crown galls from the field contain various organisms in addition to the pathogene and that transfers from colonies typical of *Phytomonas tumefaciens* vary greatly in their pathogenicity (*17*). Each of the organisms just mentioned is of a slime-producing type exceedingly difficult to separate in pure culture by the ordinary agar-plate method. These and other considerations seemed to cast doubt upon the purity of the cultures used in 1926 and led to further efforts toward purification.

In questioning the purity of the cultures used in 1926 the writers do not wish to leave out of account the possibilities of variation in the progeny of pure cultures of bacteria. However, it seems unwise to ascribe variations in cultural characters to variability of the species, unless the best available methods for purifying the cultures have been

[7] The leadership in the bacteriological studies reported in this paper was carried by W. H. Wright, who died very suddenly when the preliminary draft of this work was almost completed. The other writers have finished minor phases of the experimental work and have completed the manuscript as nearly as possible according to Professor Wright's plan.

employed. It appears that the questions which the present writers have raised regarding the purity of their own earlier cultures may also apply to the cultures of other workers on crown gall who have relied upon the simple poured-plate method for purification. Because of the questionable purity of the cultures used in 1926, the results of the bacteriological studies made upon them are not given in detail.

The purification of the cultures at hand proved to be a difficult task. An attempt was made in 1927 to secure pure cultures by the isolation of single bacterial cells. The method of Wright and McCoy (*32*) was employed. Over 400 individual cells were isolated without serious difficulty, but less than 1 per cent of them grew. Consequently, this method was given up temporarily in favor of a poured-plate method in which special precautions were taken.

Each culture to be purified was transferred to a favorable liquid medium where it was hoped that the individual cells would grow separately rather than in aggregates held together in slime. Growth was found to be active in 18 to 20 hours after transfer, before any clouding of the medium appeared. Cultures for making dilution plates were used at this stage only. Some of the same kind of liquid medium in which the organisms were growing was placed in each of the series of Petri dishes to be used for different dilutions. The dilutions were made in the liquid medium in the Petri dishes so as to avoid the clumping effect sometimes produced by agar. The agar medium was then added and the plates were incubated at room temperature. Only plates which showed less than 50 colonies were used for making subcultures. Before the typical colonies were selected they were examined under the microscope for evidence of mixture of two colonies. Transfers were finally made to agar slants, where the growth characters were observed. This procedure was carried out three successive times with clear potato-glucose agar, and a fourth time with Kellerman's (*8*) nitrogen-poor Congo-red agar. The absorption of Congo red by all the colonies was not uniform after two weeks at 21° C. Notwithstanding this difference, reisolations were made from the best dilution plates of all strains. Thirty-one strains of the crown-gall and hairy-root organisms and 10 of *Bacillus radiobacter* were purified in this way. Data concerning the origin and pathogenicity of the purified cultures are summarized in Table 3. Because of the difficulty of handling so many cultures for bacteriological studies, 18 of these cultures, which had been replated four successive times, were selected, as follows: Crown gall T–3–1c, T–5–1, T–5–3, T–6–1e, and T–35–1a; hairy root, T–32–2a, T–32–1b, T–34–1a, T–37–1b, T–38–1a, T–41–2b, T–42–1a, T–46–1b, T–48–1a, and T–49–1a; and *B. radiobacter*, R–3, R–11, and R–16. Cultures R–11 and R–16 were originally secured through the courtesy of N. R. Smith of the United States Department of Agriculture. T–5–1 and T–5–3 were the progeny of single-cell isolations.

The 18 cultures selected were compared in respect to morphology, colony characters, growth in various media, growth in litmus milk, growth on potato, limiting hydrogen-ion concentration for growth, absorption of dyes, rate of growth, nitrate reduction, fermentation of carbohydrates and related substances, and serological properties.

The cultures used for seeding the media in the various experiments were grown on clear potato-glucose agar slants for three or four days at room temperature. Equal quantities of suspensions, as uniform

as feasible, were always used in seeding the different series of culture media. Each strain of bacteria was run in duplicate in all of the experiments. All experiments, except the chemical analyses of the carbohydrate and alcohol fermentations, were repeated at least three times.

Long incubation periods were used in most of the experiments so that late changes would not be so likely to escape observation. This was done because preliminary experiments showed that the common periods of four to seven days were insufficient to bring out some of the important distinctive characters, especially those in litmus milk. All cultures were grown at room temperature unless otherwise noted.

The methods employed in making the different determinations, unless otherwise indicated, were those described in the Manual of Methods of the Society of American Bacteriologists (*26*).

MORPHOLOGY

The three organisms under consideration are typically small rods. Measurements made under standard conditions with a filar micrometer on organisms from 48-hour-old cultures on nutrient agar in smears stained with carbol fuchsin have given these figures: Crown-gall organism, 0.75–2.25μ by 0.30–1.05μ, with an average of 1.43 by 0.60μ; hairy-root organism, 0.55–2.59μ by 0.15–0.75μ, with an average of 1.44 by 0.43μ; *Bacillus radiobacter*, 0.30–2.25μ by 0.15–0.75μ, with an average of 1.12 by 0.49μ. No particular significance is attached to the differences in the measurements of these organisms.

Examinations made of water mounts from 2-weeks-old cultures and by the use of Möller's method have both failed to reveal spores. In addition old cultures have been treated by heating for 10 minutes at 85° C., after which transfers to both nutrient broth and potato-glucose agar slants showed no growth. Spores have never been found in any of the cultures.

Capsules have been demonstrated on the crown-gall and hairy-root bacteria and on *Bacillus radiobacter* with Gins's method (*3*). Abundant slime was found in cultures of these organisms and some difficulty was experienced before definite capsules were demonstrated.

With both Loeffler's and Casares-Gil's methods, each *Bacillus radiobacter* and each hairy-root organism was shown to bear a single polar flagellum. *Phytomonas tumefaciens* has yielded variable results with respect to the staining of flagella. Strains T–3–1c and T–6–1e, which were not of single-cell origin, had single polar flagella. However, repeated trials failed to demonstrate flagella on strains T–5–1 and T–5–3, which were progenies of single-cell isolations. None of the crown-gall organisms were found to be definitely motile in hanging-drop cultures. However, the hairy-root organisms were distinctly motile in hanging-drop cultures and *B. radiobacter* was actively motile.

Staining reactions showed all the cultures of crown gall, hairy root, and *Bacillus radiobacter* to be Gram-negative and not to be acid-fast.

COLONY CHARACTERS

All of the cultures were grown in meat-extract peptone broth, agar, and gelatin. Although they grew well in these media, there was very little difference in the appearance of the colonies or streaks of the three organisms. Clear potato agar containing 1 per cent of glucose gave excellent growth but did not differentiate them. The cultural

characters of colonies of each of the three organisms on this medium at 21° C. for seven days were as follows:

Surface type: Growth, rapid; form, circular (the size varied from 2 to 6 mm.); surface, smooth, shining; elevation, convex; edge, smooth, entire; internal structure, finely granular; optical characters, opaque to translucent, in some cases almost white.

Deep type: Form, mostly lenticular; internal structure, dense, nearly amorphous.

Bacillus radiobacter grew even more rapidly at first than either the crown-gall or the hairy-root strain. The colonies of *B. radiobacter* were often larger, nearly capitate, and more translucent. However, colonies of this organism were often convex, nearly white, and indistinguishable from colonies of the crown-gall organism.

GROWTH IN VARIOUS CULTURE MEDIA

In ordinary liquid media, such as plain and sugar broths, the growth characters of the three organisms were very similar. After 10 days or two weeks at 21° C. growth was shown by the formation of a heavy pellicle and a precipitate. This was more characteristic of the crown-gall and *Bacillus radiobacter* strains than of the hairy-root strains. The last named had a tendency to form less pellicle and sediment, with a more uniform clouding of the liquid. This difference was not well enough marked in these media to be of definite differential value.

Many synthetic media were tried with the anticipation that better cultural differentiation would be secured. One of the best for this work was a glycerophosphate medium devised by Lepierre (*10*) and used for a comparative study of several species of soil bacteria by Stapp and Ruschmann (*27*). This medium, with others, was altered in various ways to be used in both liquid and solid forms. The agar used in the solid media was washed in several changes of distilled water until the water-agar mixture showed no bacterial growth after standing in the 37° C. incubator for several days.

A peptone-salt medium was also found to be very satisfactory for growth of all the strains. Nine modifications of these two kinds of media, made according to the following formulas, were employed.

Medium I.—Glycerophosphate medium (Stapp and Ruschmann, *27*): Mannitol 20.0 gm.; potassium nitrate (KNO_3), 5.0 gm.; sodium chloride (NaCl), 3.8 gm.; potassium chloride (KCl), 0.1 gm.; magnesium chloride ($MgCl_2.6H_2O$), 1.0 gm.; magnesium sulphate ($MgSO_4.7H_2O$), 0.6 gm.; calcium glycerophosphate, $Ca(C_3H_5(OH)_2.HPO_4)_2$, 0.8 gm.; and distilled water, 1,000 c. c.

Medium II.—Same as Medium I, except that sodium glycerophosphate replaced calcium glycerophosphate.

Medium III.—Same as Medium I, except that potassium nitrate was omitted.

Medium IV.—Same as Medium II, except that potassium nitrate was omitted.

Medium V.—Same as Medium I, except that glucose was substituted for mannitol.

Medium VI.—Same as Medium I, except that no carbohydrate was used.

Medium VII.—Peptone-salt medium: Xylose, 5.0 gm.; magnesium sulphate ($MgSO_4.7H_2O$), 0.20 gm.; sodium chloride (NaCl), 0.2 gm.; calcium sulphate ($CaSO_4$), 0.1 gm.; dipotassium phosphate (K_2HPO_4), 0.2 gm.; peptone, 5.0 gm.; and distilled water, 1,000 c. c.

Medium VIII.—Same as Medium VII, except that glucose was substituted for xylose.

Medium IX.—Same as Medium VII, except that no carbohydrate was used.

All of the crown-gall, hairy-root, and *Bacillus radiobacter* strains were grown in the nine liquid media for 21 days at 21° C. Duplicate tubes containing 10 c. c. of medium were seeded with uniform suspen-

sions of the bacteria from cultures which had been grown on potato-glucose agar slants for two days at 21°. At the end of the incubation period each pair of duplicates was compared with a sterile control tube in respect to the following characters: Reaction (pH and total titratable acid), growth, pellicle, veil, and sediment. The summarized cultural characters of the three groups of cultures in four of these media are given in Table 5.

The most important differences between the three types of cultures (Table 5) are amount and type of growth in Media I, II, V, and VII. The 10 hairy-root strains were able to grow very little, if any, in the different glycerophosphate media. The crown-gall and *Bacillus radiobacter* strains made abundant growth in these media when potassium nitrate was present, but did not grow in Media III and IV, from which nitrogen was absent. They were likewise unable to grow when carbohydrates were omitted from Media V and VI. Neither the crown-gall nor *B. radiobacter* strains when grown in the glycerophosphate media showed significant change in pH values or titratable acidity.

TABLE 5.—*Development of the crown-gall and hairy-root organisms and Bacillus radiobacter in glycerophosphate and peptone-salt liquid culture media, after 21 days at 21° C.*

Culture groups	Cultural characters	Glycerophosphate media [a]			Peptone-salt medium [a]
		I $Ca(C_3H_5(OH)_2.HPO_4)_2$ with mannitol	II $NaC_3H_5(OH)_2.HPO_4$ with mannitol	V $Ca(C_2H_5(OH)_2.HPO_4)_2$ with glucose	VII With xylose
Crown gall, 5 strains	Control, pH	7.0	7.0	6.4	6.4
	Cultures, pH	7.4	7.4	7.2	6.3
	Control, total acid [b]	.05	.05		.3
	Cultures, total acid [b]	.05	.05		.35
	Growth [c]	++	++	++	+++
	Pellicle [c]	++	+++	++	++
	Veil [c]	+	+	+	++
	Sediment [c]	++	+	+	++
Hairy root, 10 strains	Control, pH	7.0	7.0	6.4	6.4
	Cultures, pH	6.8	7.0	6.4	4.2
	Control, total acid [b]	.05	.05		.3
	Cultures, total acid [b]	.05	.05		.62
	Growth [c]	0	0	0	++
	Pellicle [c]	0	0	0	+
	Veil [c]	0	0	0	0
	Sediment [c]	0	0	0	+
B. radiobacter, 3 strains	Control, pH	7.0	7.0	6.4	6.4
	Cultures, pH	7.6	7.6	7.4	6.4
	Control, total acid [b]	.05	.05		.3
	Cultures, total acid [b]	.05	.05		.4
	Growth [c]	+++	+++	+++	+++
	Pellicle [c]	+++	+++	++	+++
	Veil [c]	+	+	+	+
	Sediment [c]	+++	++	+	+++

[a] Roman numerals refer to media described under the same designations in the text.
[b] The total acid is given in cubic centimeters of N/10 NaOH necessary to make 10 c. c. of medium neutral to phenolphthalein.
[c] The amount of development is indicated as follows: 0, None; +, trace; ++, moderate; +++, abundant.

The growth of the three groups of organisms in the peptone-salt medium (VII) showed definite differences in amount and type. The abundant growth of the crown-gall organism and *Bacillus radiobacter*, with a pellicle, veil, and sediment, was in marked contrast to the

growth of the hairy-root strains, which was moderate and generally with little or no pellicle, veil, or sediment. (Fig. 4, H, I, and J.) No acid formation in the peptone-salt medium was caused by the crown-gall organism or *B. radiobacter*, even when glucose was present. In these cultures ammonia formation occurred and the reaction became slightly more alkaline. The hairy-root cultures, on the other hand, although showing less growth, and that of a different kind, caused a pronounced drop in the pH of the medium when glucose was present, even though some ammonia was produced. The responses of the three organisms in Medium VIII were similar to those in Medium VII. In Medium IX the hairy-root bacteria made slightly less growth than either the crown-gall organism or *B. radiobacter*.

The failure of the hairy-root strains to grow well on liquid glycerophosphate media was further tested with the same media containing agar. The liquid media were converted to solid by using 15 gm. of washed agar per liter. The agar was washed in many changes of water during two days at 37° C. The glycerophosphate agar was used with mannitol and with glucose. Streak cultures of each of the strains of the crown-gall and hairy-root organisms and *Bacillus radiobacter* were made upon the glycerophosphate agar containing mannitol and glucose. The cultures were grown as streaks in Petri dishes for 21 days at 21°. The differences shown by the three groups of organisms in the liquid media were even more conspicuous in the agar plate cultures. The crown-gall and *B. radiobacter* cultures grew well upon calcium glycerophosphate agar with mannitol, while the hairy-root strains were almost completely inhibited. (Fig. 4, K, L, and M.) The growth of the strains of *B. radiobacter* was usually heavier, raised, and more slimy than the growth of the crown-gall cultures. It was also usually surrounded by a halo of deep-brown pigment and an outer zone of a white precipitate. Streak cultures on calcium glycerophosphate agar, containing glucose and brom-thymol blue, brought out the same differences between the hairy-root strains and the others. (Fig. 4, N, O, and P.) The growth of the crown-gall and *B. radiobacter* cultures was not as abundant as with mannitol in the medium. The hairy-root strains were practically inhibited. The absorption of the dye by strains of *B. radiobacter* was greater than by those of crown gall. After about 10 days or 2 weeks a well-defined blue halo around the streaks clearly indicated an alkaline reaction.

GROWTH IN LITMUS MILK

Litmus-milk cultures of all of the strains were run in duplicate four times. In each instance the cultures were examined three times at intervals of one week. Variation in the growth characteristics of the crown-gall organism, when grown in litmus milk, has been reported by so many investigators that special care was taken to keep conditions constant.

Fresh skim milk containing 0.06 per cent of soluble litmus per liter was used for preparing the medium. Transfers were made with comparable suspensions of the bacterial growth from 48-hour-old agar plants, and the milk cultures were incubated at 28° C. The repeated trials with the three groups of organisms gave constant results.

The growth of all three groups of cultures was practically identical during the first week. The serum zone described by Löhnis and Han-

sen (*12*) as characteristic of *Bacillus radiobacter* cultures was produced also by the crown-gall and hairy-root organisms. Accompanied by pellicle formation it began to develop in all of the cultures in three or four days. After one week the serum zone was well-developed and varied from 3 to 6 mm. in depth. The color of the milk below the serum zone showed little alteration until near the end of the first week. At this time the litmus began to show a change of color and after two more weeks it appeared to be somewhat reduced. The crown-gall and the *B. radiobacter* cultures began to change toward an alkaline reaction while the hairy-root cultures were becoming acid.

After three weeks there was a well-developed difference between the crown-gall and the *Bacillus radiobacter* cultures on the one hand and the hairy-root cultures on the other. The serum zone continued to deepen in the case of the crown-gall and the *B. radiobacter* cultures, while it had a tendency to become shallower or to disappear altogether in the case of the hairy-root cultures. (Fig. 4, E, F, and G.)

A careful check of the cultures with brom-thymol blue, at the end of three weeks' growth, showed the crown-gall and the *Bacillus radiobacter* cultures to be decidedly alkaline and the hairy-root cultures quite acid. This decided difference in the growth of crown-gall and hairy-root organisms appears to be characteristic of them. Smith et al. (*25*) state in regard to the crown-gall organism, "Litmus milk is blued (never reddened) and the litmus is frequently reduced." In the present work this was found to be a consistent character of carefully purified crown-gall and *B. radiobacter* cultures. Pure cultures of the hairy-root organism, on the other hand, have always produced an acid reaction.

GROWTH ON POTATO

Growth of the three types of organisms on plain potato plugs, although not as characteristic as in litmus milk, showed that the hairy-root cultures grow differently from the crown-gall cultures. *Bacillus radiobacter* and the crown-gall organism showed a tendency to grow in a raised slimy mass along the needle track and between the sides of the tube and the potato piece. This was more characteristic of the *B. radiobacter* than of the crown-gall cultures. Browning of potato typical of *B. radiobacter* was observed also in the crown-gall cultures. Some of the *B. radiobacter* cultures seemed to cause more of this type of change than others.

Growth of the hairy-root strains on potato was watery, moderate to scant, and almost transparent. There was not the piling up or accumulation of a mass of growth such as was produced by the other types.

All these groups of organisms grew abundantly in the water under the potato plugs. The crown-gall organism and *Bacillus radiobacter* formed much more of a pellicle and precipitate in the water under the potato plugs than did the hairy-root organism.

LIMITING HYDROGEN-ION CONCENTRATION FOR GROWTH

The effect of hydrogen-ion concentration on growth was studied by varying the reaction of the following medium (*31*): Glucose, 5.0 gm.; magnesium sulphate ($MgSO_4 \cdot 7H_2O$), 0.2 gm.; sodium chloride ($NaCl$), 0.2 gm.; calcium sulphate ($CaSO_4$), 0.1 gm.; dipotassium

phosphate (K_2HPO_4), 0.2 gm.; 10 per cent yeast infusion, 100 c. c.; distilled water, 900 c. c. This medium was prepared in quantities of several liters at a time and then divided into smaller quantities for each pH value to be used. Each lot of culture media was sterilized at pH 7.0 and then adjusted to the pH value desired by the addition of N/20 hydrochloric acid under aseptic conditions. The quantity of hydrochloric acid to be added to each lot of medium was first determined for a 100 c. c. sample of the same medium. After addition of the required quantity of acid each lot of culture solution was tubed under aseptic conditions. All of the various strains of the three groups of organisms were grown in duplicate in the culture solutions of each pH value. The technic of seeding these cultures was standardized by making water suspensions of the various strains, using organisms from 2-day-old cultures on potato-glucose agar slants. After thorough shaking 0.2 c. c. portions of these suspensions were used for seeding the cultures. At the time of transfer a careful potentiometric check of the pH value of each lot of culture media was made. The cultures were first grown in solutions decreasing from pH 7.0 to pH 4.0 in steps of 0.5 pH. In these solutions growth of all strains was abundant down to pH 5.0, and decreased somewhat but was still well marked at pH 4.5. A second series of cultures was prepared in the same manner as the first except that they were adjusted to the following reactions: pH 4.6, 4.4, 4.2, 4.0, 3.8, and 3.6. The final reaction was checked potentiometrically in each case, as before. Final results as to growth were recorded after two weeks at 21° C. The critical reaction (pH) for all three groups of organisms was between pH 4.4 and 4.0. The crown-gall and *Bacillus radiobacter* cultures showed a greater tendency to stop growth abruptly at pH 4.4, while five strains of the hairy-root organisms persisted in growing somewhat at pH 4.0. It was evident that there was not a great difference in the ability of the three groups of organisms to tolerate acid. The critical hydrogen-ion concentration was about pH 4.3.

ABSORPTION OF DYES

The absorption of Congo red by *Phytomonas tumefaciens* was observed by Kellerman (*8*) and later confirmed by Smith (*24*). In an earlier part of this paper reference was made to the use of Congo-red media in replating the cultures used in this work.

Yeast-infusion mannitol agar, to which was added an aqueous solution of Congo red to make a final concentration of 1 gram to 40,000 c. c., was used for the absorption tests. The various strains of the three groups of organisms were studied as ordinary colonies in loop dilution plates and as giant colonies in plate cultures. The dilution plates and giant colonies were made from suspensions of the organisms which had grown for three days on yeast-infusion mannitol-agar slants. The plates were incubated, under favorable moisture conditions, for 14 days at 21° C. The same differences were again observed as when the cultures were plated for purification. In general the three groups of organisms absorbed the dye in the following manner: Crown gall, strong; hairy root, moderate; and *Bacillus radiobacter*, none or a trace. As judged by intensity of coloring, the most absorption occurred in the crown-gall colonies from single-cell cultures. At first the colonies showed deep pink to red centers, but after two weeks, when the giant colonies had reached a maximum

size of approximately 20 mm., the coloring was uneven. In some cases concentric rings of color and in others radial sectors of deep red were plainly visible.

Absorption of Congo red by the smaller colonies in the loop dilution plates was not always distributed evenly throughout. Some showed intense red or pink centers while others were more uniformly colored. The subsurface colonies usually exhibited more pronounced shades, often an intense red.

Both giant and dilution-plate colonies of the hairy-root organisms showed much less tendency to absorb the dye. In some cases an accumulation of dye could be noticed in the central portions of the surface colonies, and deep colonies were often distinctly pink. The red color observed in the crown-gall colonies was not seen, although some of the pinkest of the hairy-root cultures were nearly as deep as the most lightly colored of the crown-gall cultures. In both cases the differences were most definite at the end of two weeks.

The three *Bacillus radiobacter* strains used for comparison absorbed very little dye during two weeks. The colonies remained translucent and slimy, the pink of the agar showing through them. Some of the deep colonies showed a more definite pink color, but none of them were distinctly red.

Brom-thymol blue, crystal violet, basic fuchsin, and methylene blue were not absorbed as selectively as was Congo red. With the exception of brom-thymol blue, the crown-gall cultures showed the strongest absorption of these dyes. When the culture medium contained glucose, and after the reaction became acid from fermentation of the carbohydrate in lightly buffered media, brom-thymol blue was quite strongly absorbed by the hairy-root strains. Variation of the reaction did not appear to affect the absorption of Congo red by the crown-gall organisms.

RATE OF GROWTH

Löhnis and Hansen (*12*) have reported that *Bacillus radiobacter* is a much faster growing organism than many of the nodule bacteria of leguminous plants. The similarity of the growth of the crown-gall and hairy-root organisms and *B. radiobacter* on favorable solid media suggested a comparison of the rate of colony growth of the three organisms.

The rate of growth of giant colonies of all the strains in each of the three groups was determined by measurement at regular intervals for two weeks. Yeast-infusion mannitol agar was used for growing the cultures, at 21° C. The final sizes and rates of growth of all three groups of organisms were very similar. The average diameters of these colonies were as follows: Crown gall, 18 mm.; hairy root, 20 mm.; and *Bacillus radiobacter*, 22 mm. The bacteria of crown gall and hairy root are of the rapid-growing type like *B. radiobacter*. The *B. radiobacter* colonies were often more raised (convex or capitate) than those of crown gall or hairy root, but in other respects very similar.

NITRATE REDUCTION

In the peptone-salt medium containing potassium nitrate and in the other media with nitrate, the crown-gall and hairy-root organisms did not reduce the nitrate. In the *Bacillus radiobacter* cultures, on the other hand, the nitrate completely disappeared. In this respect

B. radiobacter appears to be different from the crown-gall and hairy-root organisms.

In the peptone-salt medium and in other peptone-containing media, the organisms of all three groups produced ammonia. The ammonia was evidently formed from the peptone, for when peptone-free media were seeded with the organisms no ammonia formation took place.

FERMENTATION OF SUGARS AND RELATED SUBSTANCES

The amount of carbohydrates fermented by different organisms has been measured in various ways. The most common method has been to measure the acid and gas produced in the fermentation. None of the organisms studied has been found to produce gas. Further-

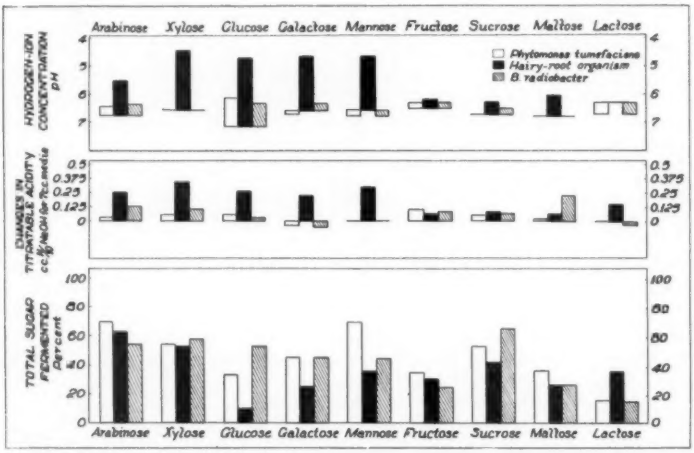

FIGURE 5.—Diagram showing the action of *Phytomonas tumefaciens*, the hairy-root organism, and *Bacillus radiobacter* on various carbohydrates as determined by hydrogen-ion concentration, changes in titratable acidity, and the percentage of total sugar fermented, as measured by quantitative sugar analyses. For each sugar fermented the hydrogen-ion concentration of the control is indicated by the continuous line through all three columns. The data on change in titratable acidity and on the percentage of sugar fermented are based, in each case, on the control as zero. The different sugars were subjected to the action of the bacteria in the peptone-salt liquid medium for three weeks at 21° C.

more, quantitative sugar determinations give a more satisfactory record of the amount of sugar fermented than can be had from records of gas formation and acidity. Consequently, in the present work it was decided to measure not only the changes in pH and titratable acidity, but also the quantitative changes in the amounts of reducing sugars present in the culture media.

The basal medium chosen for the studies on the fermentation of different carbohydrates was Medium VII, without xylose, which has been described. This medium was selected because it was favorable for the growth of all the strains of crown gall, hairy root, and *Bacillus radiobacter* employed. Five liters of this basal medium was made up to double strength. This was measured in 225 c. c. portions, placed in flasks, and autoclaved at a pressure of 15 pounds for one hour. The various carbohydrates employed were made up to 1 per cent strength in 225 c. c. portions of distilled water and autoclaved in the

same way. A flask containing 1 per cent carbohydrate solution was flamed and poured into a flask of the sterile double-strength basal medium. This mixture, containing the correct concentration of the basal medium and 0.5 per cent carbohydrate, was then transferred aseptically, in 10 c. c. portions, into sterilized test tubes. The media were incubated for a week to test sterility. The tubes containing 10 c. c. portions of the medium were seeded, respectively, in duplicate with the 18 strains of bacteria under study. Four tubes remained without seeding and were used as controls. The cultures were incubated at 21° C. for a period of 21 days before the carbohydrate fermentation was measured.

The fermentation of the carbohydrates was measured (1) by change in titratable acidity, (2) by change in pH, and (3) by quantitative determinations of the sugar in the cultures by the method described by Stiles, Peterson, and Fred (*28*). A list of the carbohydrates employed and a summary of the results of carbohydrate fermentation appear in Table 6. The action on reducing sugars is shown in Figure 5.

TABLE 6.—*Action of the crown-gall and hairy-root organisms and Bacillus radiobacter on different carbohydrates, as shown by quantitative determinations of reducing sugars, by changes in hydrogen-ion concentration, and by changes in titratable acidity*

Carbohydrate	Sugar fermented[a] by—			pH concentration with—				Change in titratable acidity[b] with—		
	Crown gall	Hairy root	B. radiobacter	Control	Crown gall	Hairy root	B. radiobacter	Crown gall	Hairy root	B. radiobacter
	Per cent	*Per cent*	*Per cent*					*C. c.*	*C. c.*	*C. c.*
Arabinose	66	60	52	6.6	6.3	5.4	6.2	+0.03	+0.23	+0.13
Xylose	52	50	55	6.4	6.3	4.4	6.4	+.05	+.32	+.10
Rhamnose				6.6	6.6	5.6	6.2	−.02	+.08	−.02
Glucose	32	[c] 9	51	7.0	6.0	4.7	6.2	+.05	+.25	+.03
Galactose	43	24	43	6.4	6.5	4.6	6.2	−.03	+.17	−.05
Mannose	66	34	43	6.4	6.6	4.6	6.6	.00	+.28	.00
Fructose	34	30	24	6.4	6.2	6.2	6.2	+.10	+.06	+.07
Sucrose	51	40	62	6.6	6.6	6.2	6.4	+.05	+.07	+.05
Maltose	35	25	25	6.6	6.6	5.9	6.4	+.01	+.05	+.19
Lactose	15	34	14	6.2	6.6	5.1	6.6	.00	+.13	−.02
Raffinose				6.6	6.8	6.8	6.8	−.07	−.12	−.05
Melezitose				6.2	6.2	6.4	6.4	−.07	−.12	−.09
Starch				6.8	8.0	8.0	8.0	−.15	−.20	−.20
Dextrin				6.2	7.2	7.3	7.4	.00	−.03	.00
Inulin				6.4	7.2	8.0	8.0	−.10	−.17	−.06
Salicin				6.2	6.1	4.6	6.2	.00	+.17	.00
Aesculin				5.8	5.8	5.8	5.8	−.10	.00	.00
Dulcitol				6.8	6.2	7.2	6.2	+.03	−.02	+.03
Mannitol				6.4	6.4	6.3	6.5	.00	.00	.00
Erythritol				6.8	7.6	4.6	7.5	−.07	+.05	−.10

[a] In each case control tubes were taken as 100 per cent.

[b] In each case the increase (+) or decrease (−) in acidity in comparison with the control tubes is recorded in c. c. N/10 sodium hydroxide. Each tube contained 10 c. c. of medium and phenolphthalein was used as the indicator.

[c] Repetition of this determination gave similar results. The low pH reading suggests that the growth of the organism was inhibited before much sugar was fermented.

The change in hydrogen-ion concentration is shown by giving the pH value of the control sample and the pH value of the culture after incubation. In the upper section of Figure 5 the continuous line across the three columns given for each sugar indicates the pH value of the control sample, while the variation from this line indicates the pH value after seeding and incubation. For example, the pH value

for the galactose control sample was 6.4, while the pH value of the medium after fermentation by the crown-gall organism was 6.5; by the hairy-root organism, 4.6; and by *Bacillus radiobacter*, 6.2. The change in titratable acidity was obtained by finding the difference between the quantity of N/10 NaOH necessary to neutralize to phenolphthalein the cultures and the controls after incubation for a period of 21 days.

An examination of the results from the fermentation studies shows several things. (1) Where sugar fermentation was determined quantitatively, crown gall and *Bacillus radiobacter* fermented approximately the same or a greater percentage of sugar than the hairy-root organism, except where lactose was used as a source of carbon. In this case the hairy-root organism fermented more of the sugar than either of the other two organisms. (2) Crown gall and *B. radiobacter* fermented the carbohydrates with but very little change in pH, while the hairy-root organism fermented arabinose, xylose, rhamnose, glucose, galactose, mannose, lactose, salicin, and erythritol with distinct decrease in pH. With these last-named carbohydrates the hairy-root organisms occasioned more change in titratable acidity than did either the crown-gall organisms or *B. radiobacter*. So far as the results of this study show, the crown-gall organism and *B. radiobacter* appear not to be differentiated from each other by means of their ability to ferment carbohydrates. Both produce fermentation with but little or no change in pH or titratable acidity. Whether these organisms are producing a neutral end product or enough ammonia from the peptone largely to neutralize any acid from the carbohydrates or are removing important ions from the medium remains to be determined. The hairy-root organism differs from the crown-gall organism and *B. radiobacter* in its ability to produce a decidedly acid condition in connection with the fermentation of certain carbohydrates.

Tests of the various strains with respect to hydrolysis of starch were made by growing streak cultures of the organisms on the basal medium for carbohydrate-fermentation studies plus soluble starch and agar. None of the cultures showed any diastatic action after 21 days at 21° C., when they were flooded with iodine solution. Eckford's method also gave negative results.

AGGLUTINATION TESTS

Agglutination tests were made of 5 strains of crown-gall bacteria, 2 of which were of single-cell origin; 11 strains of the hairy-root organisms; and 3 strains of *Bacillus radiobacter*. The rapid method described by Noble (*15*) and modified by Huddleson and Carlson (*6*) was used.

Cultures of the organisms for immunization of animals and for antigens were grown on neutral clear potato agar without glucose for four days. Rabbits were immunized with suspensions of these organisms in 0.85 per cent NaCl solution. The suspensions were made by washing the bacterial growth free from the agar, shaking, filtering through glass wool, and standardizing to a turbidity which would conceal a 2-mm. loop made from No. 26 B. & S. gauge wire at a depth of 12 mm.

Rabbits were injected intraperitoneally with the suspensions at intervals of four or five days until four injections had been used.

The amounts of injections were, respectively, 0.5, 1.0, 3.0, and 5.0 c. c. A week after the last injection each rabbit was bled from an ear vein. Since the test of the titer was sufficiently high in each case, the animal was bled from the ear until sufficient blood was secured for the agglutination tests. After coagulation of the blood in the ice box the serum was removed and preserved for the agglutination tests.

The heavy antigen was prepared in the same manner as the suspensions used for inoculation of the animals, except that the bacteria were suspended in 12 per cent sodium chloride, and phenol was added until the mixture contained 0.5 per cent. The heavy suspensions were made up to a turbidity which at a depth of 8 mm. concealed the 2-mm. loop made from No. 26 B. & S. gage wire.

Dilutions of antisera, 1:50, 1:100, 1:200, 1:500, 1:1,000, and 1:2,000, were used in preliminary trials. Agglutinations with homologous sera showed a titer of 1:2,000 in practically every case. The final agglutinations were run by making dilutions of 1:500, 1:1,000, and 1:2,000 with a calibrated capillary pipette. The results are summarized in Table 7.

One of the hairy-root cultures was omitted from the serological tests because it had failed to make satisfactory growth when the antigens were prepared.

Each of the 19 strains of the three groups of organisms employed was agglutinated by the antiserum from its own group but not that from any other group except in the case of two strains of the crown-gall bacteria, one of the hairy-root organism, and one of *Bacillus radiobacter*. None of these four aberrant strains was of single-cell origin. Of the two aberrant strains of the crown-gall organism one gave no agglutination with any of the sera, and the other cross agglutinated completely with the hairy-root organism and with neither the crown-gall organism nor *B. radiobacter*. In various tests this latter strain appeared to be a mixture of the crown-gall and hairy-root organisms, as explained in the footnote to Table 7. The one aberrant strain each of the hairy-root organism and *B. radiobacter* gave no agglutination with any of the antisera.

TABLE 7.—*Results of agglutination tests with cultures of the crown-gall and hairy-root organisms and Bacillus radiobacter*

Antigens		Results with sera from—		
Culture group	Number of strains used	Crown gall	Hairy root	Bacillus radiobacter
Crown gall	3	+	0	0
	1	0	0	0
	1	0	*+	0
Hairy root	10	0	+	0
	1	0	0	0
Bacillus radiobacter	2	0	0	+
	1	0	0	0

* In these tests and also in litmus milk, glycerophosphate media, and in the fermentation of xylose, rhamnose, galactose, mannose, lactose, and salicin, the behavior of this culture indicates a mixture of the crown-gall and hairy-root organisms.

STUDIES ON THE PROGENY OF SINGLE CELLS

An examination of the results secured with the cultures purified by four successive replatings (Table 3) shows that some of the original cultures were mixed. It also appears that even after the unusual precautions taken to purify these cultures by the poured-plate method, 2 of the 15 crown-gall and hairy-root strains (T–35–1a and T–38–1a) employed for bacteriological studies were mixed cultures. In the bacteriological studies and the inoculation work, the responses of each of these strains were in part typical of the crown-gall organism and in part typical of the hairy-root organism. For some time it was considered that both of these strains might be pure cultures which varied from the usual type and were perhaps intermediate between the typical crown-gall and hairy-root organisms, as they showed mixed characters. In order to gain further evidence on this point, single-cell cultures were isolated, according to the methods of Wright and McCoy (*32*) and Wright and Nakajima (*33*) from strain T–38–1a and subjected to bacteriological studies and inoculation tests. The characters of this strain were somewhat less clearly those that might be expected from a mixed culture of the crown-gall and hairy-root organisms than were those of strain T–35–1a. As time was not available to analyze both strains, T–38–1a was chosen as seemingly the more perplexing one. Four single-cell cultures were isolated. One of these consistently showed the typical characters of the crown-gall organism, while all the others consistently showed the typical characters of the hairy-root organism. A more detailed account of this work is reported by Wright, Hendrickson, and Riker (*34*).

Even when the single-cell technic is used, certain questions may be raised concerning the quality and purity of the cultures.

(1) When only an occasional isolated cell would grow, as was the case in the earlier stages of development of the technic, it was questionable whether the few cells that grew were satisfactorily representative of the large majority that did not. The improvement of the technic to the point where the majority of the isolated cells grow appears to minimize any element of doubt that these cells may be regarded as satisfactory random samples of the population from which they are taken.

(2) The possibility that an unrecognized or ultramicroscopic stage of another microorganism might be carried along with the single cells deserves consideration. However, this possibility was greatly reduced by selecting the single cells from media at a time when growth conditions were most favorable for the development of the usual vegetative forms.

(3) Attention should also be given the further possibility that some ultramicroscopic form of life might be transferred along with the single cells. All bacterial cultures are subject to this question, and it appears that they must continue to be unless biological science develops methods that will completely insure the detection of any living thing which may exist as a contaminant. In view of the lack of such technic at present, it seems desirable to seek to apply the best available methods to the purification of cultures and to evaluate the probability of their purity as critically as possible.

Apart from the technic of isolation, the best available criterion of the purity of single-cell cultures appears to be their behavior. If this is erratic, it remains questionable whether the variability results from genetic plasticity of the strain or from impurity of the culture. If, on the other hand, there is unusual consistency in the behavior of the strain throughout a sufficiently exacting morphological and physiological study, the chances of contamination or admixture appear to be minimized. Under the conditions thus far employed, the single-cell strains studied have shown a striking consistency in behavior. It remains for further work to reveal their behavior under diverse conditions. This technic seems to offer a satisfactory basis upon which to study variability of the organisms under investigation. In view of the extreme difficulty experienced in consistently separating the crown-gall and hairy-root organisms from each other and from various similar bacteria, it would appear that any report of variability within this group of organisms which is not based upon single-cell isolations is open to question. The work on the present writers' cultures with the single-cell technic has been reported by Wright, Hendrickson, and Riker (*34*). Eight strains of single-cell origin from the crown-gall organism and nine of like origin from the hairy-root organism were studied. No single-cell strain showed mixed characters of the hairy-root and the crown-gall organisms. In every case the crown-gall organism has given the typical crown-gall reactions and the hairy-root organism the typical hairy-root reactions. While variations may very possibly be found by study of a larger number of cultures, those studied thus far have behaved very consistently.

IDENTITY AND TECHNICAL DESCRIPTION OF THE HAIRY-ROOT ORGANISM

The differential characteristics of *Phytomonas tumefaciens*, of the hairy-root organism, and of *Bacillus radiobacter* are shown in Table 8. The considerable number of characters which distinguish the hairy-root organism from the crown-gall pathogene shows that the two organisms are different. While the definition of a bacterial species is so much a matter of debate, the present writers hesitate to describe a new species. However, convenience, which is a primary requisite of a workable classification, demands that the hairy-root organism be given some designation. If it is considered a variety of the crown-gall organism and the classification is based chiefly on morphological and cultural characters, the difficulty arises that *B. radiobacter* and probably a number of other mucoid soil organisms should be included in the same species. If this were done, all these organisms, including those that cause crown gall and hairy root, would seem to be referable to *B. radiobacter*, this organism having been described prior to the others. The objections to discarding such a well-known and firmly established species as *Phytomonas tumefaciens* seem quite obvious. If, on the other hand, the responses of plants to inoculations are regarded as differential characters of specific rank, it would seem unnecessary to refer the crown-gall organism to *B. radiobacter*, and the crown-gall and hairy-root organisms and *B. radiobacter* would fall into three species.

TABLE 8.—*Differential characteristics of Phytomonas tumefaciens, the hairy-root organism, and Bacillus radiobacter*

Differential characters [a]	P. tumefaciens [b] (5 strains)	Hairy-root organism (10 strains)	B. radiobacter (3 strains)
Reaction on:			
Apple	Causes crown gall	Causes hairy root	No reaction.
Tomato	do	Slight or no reaction	Do.
Motility	Motility doubtful	Motile	Actively motile.
Growth on calcium glycerophosphate medium with mannitol.	Abundant	None to trace	Abundant, with brown pigment.
Action on litmus milk	Deep serum zone; Grayish brown; Neutral	Slight serum zone; Litmus reduced; Acid	Deep serum zone. Grayish brown. Neutral.
Absorption of:			
Congo red	Strong	Slight	Very slight.
Brom-thymol blue	Slight	do	Strong.
Nitrate reduction	No nitrite	No nitrite	Nitrate disappears.
Action on:			
Arabinose and xylose	Slightly acid; Strong fermentation [c]	Strongly acid; Strong fermentation [c]	Slightly acid. Strong fermentation.[c]
Glucose	Slightly acid; Medium fermentation [c]	Strongly acid; Weak fermentation [c]	Slightly acid. Strong fermentation.[c]
Galactose, fructose, and mannose.	Slightly acid; Medium to strong fermentation. [c]	Strongly acid; Medium to strong fermentation. [c]	Slightly acid. Medium to strong fermentation.[c]
Lactose	No acid; Weak fermentation [c]	Strongly acid; Medium fermentation [c]	No acid. Weak fermentation.[c]
Maltose	No acid; Medium fermentation [c]	Slightly acid; Medium fermentation [c]	Slightly acid. Medium fermentation.[c]
Salicin	Slightly acid	Strongly acid	No acid.
Erythritol	Slightly alkaline	do	Slightly alkaline.
Agglutination	In own antiserum only [d]	In own antiserum only [d]	In own antiserum only.[d]

[a] All strains of each group gave the same result unless otherwise noted.
[b] Two of the five strains used throughout this work were secured from single-cell isolations. The reactions of T-35-1a, which is not a single-cell strain, are not included in this summary because this culture appears to be a mixture of crown-gall and hairy-root organisms.
[c] Amount of sugar fermented was ascertained by quantitative sugar determinations.
[d] Exceptions are noted in Table 7.

In view of the very considerable differences in bacteriological characters and host responses found between the hairy-root and crown-gall organisms, and the ample precedents for making a new species of an organism which is so distinct in a number of characters, it appears more useful, in the opinion of the present writers, to treat the hairy-root organism as a new species. The hairy-root organism, as described in detail in an earlier section of this paper, is therefore named *Phytomonas rhizogenes*, n. sp. Synonyms according to other systems of classification in common use among plant pathologists are *Bacterium rhizogenes*, n. sp. and *Pseudomonas rhizogenes*, n. sp. A brief description follows.

Phytomonas rhizogenes, n. sp.

Short nonspore-forming rods, 0.55–2.59μ by 0.15–0.75μ, average 1.44μ by 0.43μ; motile by means of one polar flagellum; capsule present; Gram-negative; not acid-fast. On clear potato-glucose agar at 25° C. for seven days the surface colonies have these characters: Growth rapid; form circular, 2 to 6 mm. in diameter; surface smooth; elevation convex; edge smooth; internal structure finely granular; optical characters, translucent through gray to almost white. Deep colonies are lenticular and dense. Little or no growth in glycerophosphate mannitol medium, liquid or solid, with potassium nitrate as nitrogen source. Acid is produced in litmus milk; the litmus is slowly reduced, and a slight serum zone is formed which later disappears. Watery, moderate to scant growth on potato plugs. No growth below pH 4.0. Absorbs Congo red and brom-thymol blue slightly. No nitrite formed from nitrate. No apparent growth in the closed arm of fermentation tubes. Acid but no gas from arabinose, xylose, rhamnose, glucose, galactose, mannose, maltose, lactose, salicin, and erythritol. No acid or gas from fructose, sucrose, raffinose, melezitose, starch, dextrin,

inulin, aesculin, dulcitol, and mannitol. Starch not hydrolyzed. Favorable temperature for growth, 20° to 28° C. Causes infectious hairy root on apple.

SUMMARY

An infectious type of hairy root on nursery apple trees is described.

A limited number of bacteriological examinations of burrknots have failed to reveal any causal organism.

Isolation studies from 96 enlargements of the hairy-root type, including "woolly knot," are recorded. This number includes the cultures employed in preliminary work. From these, 367 cultures were used for inoculation studies, which involved 4,252 inoculations into tomato, tobacco, and apple. Four of the 96 enlargements were found to contain the crown-gall organism, 1 yielded cultures that were apparently mixtures of the crown-gall and hairy-root organisms, 13 yielded no pathogenic organisms, and 78 yielded the hairy-root organism.

Attempts were made to reisolate the hairy-root organism from 40 hairy-root specimens induced by inoculation. All yielded typical cultures. Cultures from 12 of these were reinoculated into apple. Typical hairy root was induced by bacteria from each of these 12 specimens.

The hairy-root organism has been inoculated into stems of apple, rose, honeysuckle, sugar beet, bean, and Paris daisy with positive results. Little or no response was induced in stems of tomato and tobacco.

In preliminary experiments cuttings or layers of certain plants treated with the hairy-root organism have rooted sooner and more vigorously than those untreated. These results suggest the possibility of using this organism to stimulate root production in the propagation of certain plants. More work is necessary before conclusions can be drawn.

The roots stimulated by the hairy-root organism have been found capable of supporting the life of apple, Delphinium, and Paris daisy plants after all the other roots were removed. Whether the effects of the hairy-root organism on the several plants over a long period are harmful or beneficial has not yet been determined.

The reactions of the underground parts of Wealthy apple stems to crown-gall inoculations, hairy-root inoculations, and needle punctures were very similar at the end of two weeks in preliminary comparative trials in Wisconsin. After a month the enlargements showed a decided increase in size and a change in surface character to the crown-gall type, while those of the controls were very little larger and showed no change in character. After two months the symptoms of hairy root were beginning to differentiate from those of crown gall, though at this stage they still might easily be confused. The controls at this time had healed over. Typical clearly differentiated hairy-root and crown-gall symptoms developed within three months.

Many of the roots which followed hairy-root inoculation were winterkilled in Wisconsin, but new roots of like type appeared the following spring. In the second year after inoculation deeply convoluted knots were often found at the places of hairy-root inoculation. These knots need further investigation.

A detailed comparative study was made of the bacteriological characters of 5 strains of *Phytomonas tumefaciens*, 10 strains of the

hairy-root organism, and 3 strains of *Bacillus radiobacter*. Their differences are summarized in Table 8.

The poured-plate method, even when repeatedly employed in successive trials and with special precautions, was found inadequate consistently to separate the crown-gall and hairy-root organisms from each other.

The bacteriological studies reported on the crown-gall and hairy-root organisms have been checked up and extended by studies reported elsewhere (*34*) on cultures which were progenies of single-cell isolations. Confirmatory results were secured.

Typical crown-gall and hairy-root cultures have been separated by the single-cell isolation technic from a culture that was earlier thought a probable variant and intermediate form.

Because of the difficulty of separating the crown-gall and hairy-root organisms, reports of variability within these species appear to be open to question when they do not deal with progeny from single-cell isolations.

The hairy-root organism is described under the name *Phytomonas rhizogenes*, n. sp.

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STUDIES ON THE PROGENY OF SINGLE-CELL ISOLATIONS FROM THE HAIRY-ROOT AND CROWN-GALL ORGANISMS[1]

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INTRODUCTION

The difficulty of separating uncontaminated strains of the crown-gall [3] and hairy-root [4] organisms and of *Bacillus radiobacter* Beij. and Van Deld.[5] has been discussed by Riker and others.[6] These writers reported that the poured-plate method was inadequate to separate the organisms of this group into pure cultures with certainty. They reached this conclusion after taking unusual care in preparing the dilutions, in pouring the dilution plates, and in making four successive isolation series from each of 31 original cultures of the crown-gall and hairy-root organisms. Some of the reasons for the difficulty in obtaining pure strains of these bacteria by the poured-plate method are (1) the mixture of organisms, including various soil bacteria, that commonly occurs in the underground parts from which the original platings are made; (2) the large amount of slime produced by the bacteria, which hinders separation of individual cells; and (3) the fact that isolated single cells ordinarily fail to grow when placed in a comparatively large quantity of medium. In repeated trials by the present writers less than 1 per cent of the single cells of these organisms grew when each was placed in 10 c. c. of sterile medium of the most favorable kind known. Consequently, when a cell becomes well separated from others in a poured-agar plate, the chances in favor of its growth seem very small. For a long time this inability to get the isolated cells to grow prevented the successful use of the single-cell technic for studies of *Phytomonas tumefaciens* and *P. rhizogenes*.

SINGLE-CELL ISOLATIONS

The improvement of the single-cell technic by Wright and McCoy [7] and later by Wright and Nakajima [8] provided a new and practical

[1] Received for publication May 1, 1930; issued October, 1930. Published with the approval of the director of the Wisconsin Agricultural Experiment Station. The investigations reported in this paper were conducted as part of a cooperative project between the Bureau of Plant Industry, U. S. Department of Agriculture, and the University of Wisconsin. This work has been supported in part by a grant from the special research fund of the University of Wisconsin.

[2] Professor Wright died May 3, 1929, when the work was almost completed. The few remaining experiments were performed and the manuscript prepared as nearly as possible in the way that he had planned.

[3] *Phytomonas tumefaciens* (Smith and Town.) Com. S. A. B.

[4] *Phytomonas rhizogenes* R., B., W., K., and S.

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method of studying the crown-gall and hairy-root organisms from cultures that were the progeny of single cells. Consequently, some of the cultures employed by Riker and his coworkers [9] were used for making single-cell isolations, as mentioned earlier. Since the same culture numbers adopted by these writers are used here, the reader is referred to their paper for the histories and the records of pathogenicity of the original cultures. The original cultures employed for single-cell isolations were as follows: Crown gall, T–5; intermediate (?) between hairy root and crown gall, T–38; and hairy root, T–32 and T–48.

The medium which seemed most suitable for the single-cell isolation work was selected after trials with a number of different media. The peptone-salt medium selected as the best had the following composition: Magnesium sulphate ($MgSO_4.7H_2O$), 0.2 gm.; sodium chloride ($NaCl$), 0.2 gm.; dipotassium phosphate (K_2HPO_4), 0.2 gm.; calcium chloride ($CaCl_2$), 0.1 gm.; peptone, 5 gm.; and distilled water 1,000 c. c. The reaction was pH 7.0.

The age of the culture at the time the single cells were picked was very important. Since cultures that are too old or too young may contain a number of organisms that are either dead or not actively growing, it was necessary to determine for each strain the time most favorable for finding rapidly dividing cells, or the "logarithmic growth phase" as defined by Buchanan.[10] This was accomplished by making counts on the cultures used, according to the method of Breed and Brew.[11] The first count was made immediately after the transfer. Subsequent counts were made after 6, 10, 14, 18, 22, and 26 hours, respectively, at 21° C. Graphs of the results of these counts are given in Figure 1. The curves show that under the conditions employed the bacteria of all the cultures were in the "logarithmic growth phase" during the period between 12 and 16 hours after transfer. Consequently this time was chosen for making the single-cell isolations.

The methods employed in isolating and cultivating the single bacterial cells were modifications of Chambers's technic as described by Wright and McCoy [12] and Wright and Nakajima.[13] Part of this modified technic is briefly as follows: Two sterile cover slips served as covers for the glass moist chamber used on the stage of the microscope. One of these carried a small drop of the culture of the desired age and the other a small drop of sterile medium. Two sterile micropipettes, with apertures approximating three times the length of the organisms to be isolated, were alternately employed in making the isolations. The edge of the drop containing the organisms was placed in the field of the microscope by moving the mechanical stage. One of the pipettes was made to touch the edge of this drop, a portion of which was drawn into the pipette by capillarity. The pipette and cover slip were manipulated so as to deposit a series of microscopic drops on the dry portion of the cover slip. The process was continued until a microscopic drop formed that was observed to contain only one cell. This microscopic drop with the single organism was then

[9] See footnote 6.

[10] BUCHANAN, R. E. LIFE PHASES IN A BACTERIAL CULTURE. Jour. Infect. Diseases 23: [109]–125, illus. 1918.

[11] BREED, R. S., and BREW, J. D. COUNTING BACTERIA BY MEANS OF THE MICROSCOPE. N. Y. State Agr. Expt. Sta. Tech. Bul. 49, 31 p., illus. 1916.

[12] See footnote 7.

[13] See footnote 8.

picked up with the other sterile micropipette and deposited on the other coverslip near the small drop of sterile medium. The microscopic drop was then examined to make certain that the one cell, and only that one, was present. With the rubber-tube attachment to

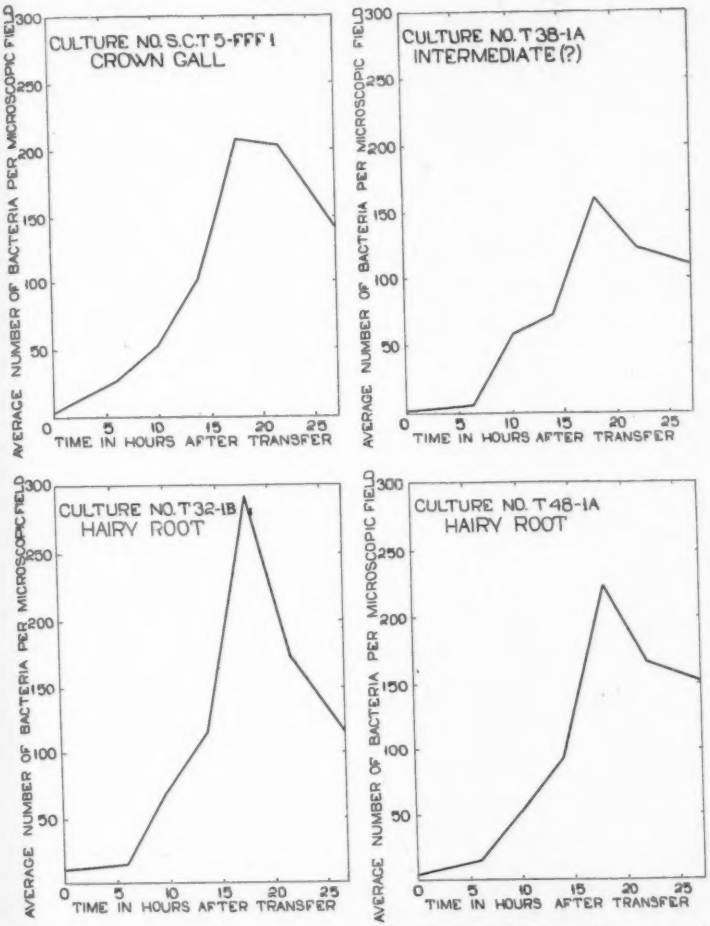

FIGURE 1.—Results secured from direct counts of the number of bacteria present in the peptone-salt medium at progressive intervals of time following transfers from the original cultures employed

the pipette the edge of the small sterile drop was blown gently around the microscopic drop containing the single cell until the two drops merged. The cover slip with the single cell in the small drop of fresh medium was removed from the moist chamber on the microscope and mounted with sterile vaseline on a sterile hanging-drop slide. A drop of sterile medium was provided at the bottom of the depression in the

slide to prevent drying. The slides prepared in this manner were incubated at about 21° C. After two to five days, when the drop showed bacterial growth, it was increased in size by adding sterile medium from a capillary pipette. By gradually increasing the amount of medium in two or three increments at suitable intervals a condition was reached which permitted transfer of the culture to a liquid or solid medium in a test tube, without fear of killing the organisms.

Usually a high percentage (up to 90 per cent in the best runs) of the single cells isolated from the crown-gall and hairy-root organisms by this technic grew. Thus far no case of contamination has been observed. This method insures the purity of the cultures as far as the present available technic permits. Although the possibility remains that the ultramicroscopic form of some other organism might be carried along with the single cell, this seems quite remote because (1) the cultures from which the cells were picked were provided with favorable conditions for the development and active growth of the microscopic forms and (2) subsequent bacteriological studies of the progenies of the single cells did not give evidence of mixture. There is also the possibility that a filterable virus might be carried along with the single cell. This possibility has been discussed by Riker and his associates [14] and will not be considered here.

With the single-cell technic described, several series of isolations were made of the typical crown-gall and hairy-root organisms. From the crown-gall culture T–5–fff1 (subculture of T–5) the following cultures were isolated: A–1, A–2, A–3, A–4, A–5, A–6, and A–7; from the culture T–38–1a (subculture of T–38)—a culture intermediate (?) between hairy root and crown gall—B–2, B–3, B–4, and B–5; from the hairy-root culture T–32–1b (subculture of T–32), C–1, C–10, C–11, C–12, and C–13; and from the hairy-root culture T–48–1a (subculture of T–48), D–20. Culture T–5–fff1 was itself of single-cell origin from T–5. Each of the other cultures from which the single cells were isolated had been replated four successive times from the original culture. The identifications of the cultures were based on inoculations on tomato and apple and on bacteriological studies, as described earlier.[14] T–38–1a, the intermediate (?) culture, produced crown gall on tomato and a mixture of crown gall and hairy root on apple. However, its bacteriological reactions were predominantly those of the hairy-root organism.

BACTERIOLOGICAL STUDIES

The bacteriological studies carried out on these single-cell cultures and their mother cultures were in each case parallel to those reported by Riker and his associates.[14] Since the present writers used the technic which these authors have fully described in their paper, only the results are recorded here.

Morphologically all the crown-gall and hairy-root cultures were small rods, approximately 0.3μ to 0.4μ by 0.5μ to 3.0μ. They were nonspore-forming, Gram-negative, and not acid-fast. Limited experiments showed that the hairy-root organism was motile by one polar flagellum. Experiments thus far conducted with the crown-gall organism have shown no flagella and no motility.

Absorption of Congo red was shown by all the cultures on the yeast-infusion glucose agar after 11 days at room temperature.

[14] See footnote 6.

However, the absorption by the crown-gall organism was, on an average, somewhat stronger than that by the hairy-root organism.

Indol was not produced, starch was not hydrolyzed, and nitrate was not reduced to nitrite by any of the crown-gall or hairy-root cultures.

The cultural characteristics of the crown-gall organism were markedly different from those of the hairy-root organism in certain media. Differentiation was secured in calcium glycerophosphate agar with mannitol. All the crown-gall cultures grew abundantly, while the hairy-root organism consistently made little or no growth. In liquid peptone and in liquid yeast-infusion glucose media the crown-gall cultures, without exception, produced a pellicle, a veil, and a slight precipitate, but no turbidity. In the same media none of the hairy-root cultures produced a pellicle or a veil, but all showed either moderate or abundant clouding with a slight precipitate. These cultures were incubated for six days at 30° C. Litmus milk also differentiated these two groups of organisms. The crown-gall organism caused this medium to turn alkaline, reduced the litmus, and produced a conspicuous serum zone at the top. The hairy-root organism produced acid, reduced the litmus, and formed a serum zone which largely or entirely disappeared after three weeks at 30° C. These results are recorded in Table 1.

Inoculation tests with each of these organisms were made on the roots of sugar beets in the greenhouse. At the end of two months each of the crown-gall cultures had induced crown gall and each of the hairy-root cultures had induced hairy root. (Table 1.) Further inoculation studies are being made on other test plants, including apple.

The single-cell analysis of the culture T–38–1a, which was earlier regarded as possibly a pure culture intermediate between the crown-gall and hairy-root organisms because it had shown a mixture of the characters of these organisms in the bacteriological studies and inoculation work, has shown consistent results. In every test employed the single-cell progenies B–2, B–4, and B–5 from this culture have behaved like the hairy-root organism. On the other hand, the single-cell progeny B–3 has consistently behaved like the crown-gall organism. These organisms apparently had not been separated, even by the unusual care taken with the four successive poured-plate isolations. These results present strong evidence that, instead of being a pure culture intermediate between the crown-gall and hairy-root organisms, T–38–1a was a mixed culture of both.

Under the conditions and over the range of characters studied, the behavior of the seven single-cell progenies, A–1, A–2, A–3, A–4, A–5, A–6, and A–7, from the crown-gall stock culture T–5–fff1, was remarkably consistent. These studies are being continued to gain further evidence concerning the range of variability of the crown-gall and hairy-root organisms.

The fermentation of carbohydrates showed a distinct difference between the crown-gall and hairy-root organisms. Determinations were made of the actions of these organisms on 0.5 per cent solutions of xylose, glucose, galactose, mannose, lactose, and erythritol in the liquid peptone medium as measured by the hydrogen-ion concentration. From each of these carbohydrates each of the hairy-root cultures produced a considerable amount of acid, while each of the crown-gall cultures produced little or no acid. The results of this work are given in Table 2.

TABLE 1.—*Growth characters in different media and reactions on sugar beets shown by single-cell strains of the crown-gall and hairy-root organisms and by their mother cultures* [a]

Culture used	Mother culture	Characters in—											Reaction on sugar beet
		Calcium glycerophosphate agar with mannitol [b]	Liquid peptone medium [c]			Liquid yeast-infusion glucose medium [d]			Litmus milk				
		Growth	Pellicle	Veil	Turbidity	Pellicle	Veil	Turbidity	Acid	Alkaline	Litmus reduced	Serum zone	
Crown gall T-5-fff1	T-5	+++	++	+	0	+++	++	0	0	+++	+++	+++	Crown gall.
Crown gall A-1	T-5-fff1	+++	++	+	0	+++	++	0	0	+++	+++	+++	Do.
Crown gall A-2	do	+++	++	+	0	+++	++	0	0	+++	+++	+++	Do.
Crown gall A-3	do	+++	++	+	0	+++	++	0	0	+++	+++	+++	Do.
Crown gall A-4	do	+++	++	+	0	+++	++	0	0	+++	+++	+++	Do.
Crown gall A-5	do	+++	++	+	0	+++	++	0	0	+++	+++	+++	Do.
Crown gall A-6	do	+++	++	+	0	+++	++	0	0	+++	+++	+++	Do.
Crown gall A-7	do	+++	++	+	0	+++	++	0	0	+++	+++	+++	Do.
Intermediate (?) T-38-1a	T-38	0	0	0	++	0	0	+++	+++	0	+	+	Hairy root.
Hairy root B-2	T-38-1a	+	0	0	++	0	0	+++	+++	0	+	+	Do.
Crown gall B-3	do	+++	++	+	0	+++	++	0	0	+++	+++	+++	Crown gall.
Hairy root B-4	do	0	0	0	++	0	0	+++	+++	0	+	+	Hairy root.
Hairy root B-5	do	0	0	0	++	0	0	+++	+++	0	+	+	Do.
Hairy root T-32-1b	T-32	+	0	0	++	0	0	+++	+++	0	+	+	Hairy root.
Hairy root C-1	T-32-1b	0	0	0	++	0	0	+++	+++	0	+	+	Do.
Hairy root C-10	do	0	0	0	++	0	0	+++	+++	0	+	+	Hairy root.
Hairy root C-11	do	0	0	0	++	0	0	+++	+++	0	+	+	Do.
Hairy root C-12	do	0	0	0	++	0	0	+++	+++	0	+	+	Do.
Hairy root C-13	do	0	0	0	++	0	0	+++	+++	0	+	+	Do.
Hairy root T-48-1a	T-48	+	0	0	++	0	0	+++	+++	0	+	+	Do.
Hairy root D-20	T-48-1a	+	0	0	++	0	0	+++	+++	0	+	+	Hairy root.

[a] The different degrees in the development of the various characters are shown as follows: 0, none; +, trace; ++, moderate; and +++, abundant.

[b] This medium contained: Mannitol, 20 gm.; potassium nitrate (KNC_3), 5 gm.; sodium chloride (NaCl), 3.8 gm.; potassium chloride (KCl), 0.1 gm.; magnesium chloride ($MgCl_2.6\,H_2O$), 1 gm.; magnesium sulphate ($MgSO_4.7H_2O$), 0.6 gm.; calcium glycerophosphate $Ca\,(C_3H_5\,(OH)_2.HPO_4)_2$, 0.8 gm.; agar 15 gm.; and water, 1,000 c. c. The reaction was pH 6.8. The agar had been washed for two days at 37° C.

[c] This liquid medium contained: Magnesium sulphate ($MgSO_4.7H_2O$), 0.2 gm.; sodium chloride (NaCl), 0.2 gm.; dipotassium phosphate (K_2HPO_4), 0.2 gm.; calcium chloride ($CaCl_2$), 0.1 gm.; peptone, 5 gm.; and distilled water, 1,000 c. c. The reaction was pH 7.0.

[d] This medium contained: Glucose, 5 gm.; magnesium sulphate ($MgSO_4.7H_2O$), 0.2 gm.; sodium chloride (NaCl), 0.2 gm.; calcium sulphate ($CaSO_4$), 0.1 gm.; dipotassium phosphate (K_2HPO_4), 0.2 gm.; 10 per cent yeast infusion, 100 c. c.; and water, 900 c. c. The reaction was pH 7.0.

TABLE 2.—*Acid production from different carbohydrates by single-cell strains of the crown-gall and hairy-root organisms and by their mother cultures* [a]

Culture used	Mother culture	Acid production from—					
		Xylose	Glucose	Mannose	Galactose	Lactose	Erythritol
		pH	*pH*	*pH*	*pH*	*pH*	*pH*
Crown gall T-5-fff1	T-5	5.8	5.6	6.0	5.4	5.8	6.8
Crown gall A-1	T-5-fff1	5.8	5.9	6.2	5.0	6.8	6.8
Crown gall A-2	do	5.9	6.0	6.1	5.0	6.8	6.8
Crown gall A-3	do	6.0	6.0	6.0	5.1	6.8	6.8
Crown gall A-4	do	6.2	5.8	6.0	5.0	6.7	6.8
Crown gall A-5	do	6.2	6.0	6.2	5.2	6.6	6.8
Crown gall A-6	do	5.9	6.8	6.1	5.1	6.2	6.8
Crown gall A-7	do	5.8	5.6	6.2	5.0	6.7	6.8
Intermediate (?) T-38-1a	T-38	4.6	4.4	4.0	4.3	4.8	5.1
Hairy root B-2	T-38-1a	4.7	4.8	4.4	4.6	4.6	4.9
Crown gall B-3	do	5.8	5.8	6.4	5.0	6.8	6.8
Hairy root B-4	do				4.2	4.4	5.9
Hairy root T-32-1b	T-32	4.7	4.7	4.8	4.3	4.5	5.1
Hairy root C-1	T-32-1b	4.4	4.8	4.5	4.2	4.8	5.0
Hairy root C-10	do	4.8	4.8	4.9	4.4	4.8	4.9
Hairy root C-11	do	4.4	4.3	4.2	4.3	4.4	4.7
Hairy root C-12	do	4.9	4.9	4.7	4.3	4.8	5.0
Hairy root C-13	do	5.0	4.8	4.9	4.4	4.5	5.0
Hairy root T-48-1a	T-48	4.7	4.7	4.5	4.4	4.8	4.8
Hairy root D-20	T-48-1a	4.3	4.6	4.4	4.3	4.2	4.6
Control		6.8	6.8	6.8	6.8	6.8	6.8

[a] The readings were taken after 12 days at 30° C. The sugars were sterilized separately and added aseptically to liquid peptone-salt medium. The formula is given in footnote c of Table 1.

SUMMARY

The poured-plate method is shown to be inadequate for the consistent isolation of uncontaminated cultures of the crown-gall and hairy-root organisms (*Phytomonas tumefaciens* and *P. rhizogenes*).

Usually a high percentage of the single cells isolated from cultures of the crown-gall and hairy-root organisms have been made to grow.

The cultures from single-cell isolations were remarkably consistent in their behavior. Those from the crown-gall pathogene uniformly gave typical reactions for that organism. Likewise, those from the hairy-root organism consistently gave typical reactions for that organism. Four single-cell isolations were made from a culture that showed a mixture of the characters of the crown-gall and hairy-root organisms and was earlier thought to be possibly a pure culture of an organism intermediate between these. In the bacteriological studies and inoculation tests one of these single-cell strains consistently showed the typical characters of the crown-gall organism and the other three consistently showed those of the hairy-root organism.

In consideration of the limitations of the poured-plate method for purifying cultures, it appears that reports of variations of the crown-gall and hairy-root organisms not based upon work with single-cell isolations are open to question.

SIZE OF LIST QUADRAT FOR USE IN DETERMINING EFFECTS OF DIFFERENT SYSTEMS OF GRAZING UPON AGROPYRON SMITHII MIXED PRAIRIE [1]

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INTRODUCTION

The question of the most suitable size of quadrat to use in analyzing herbaceous vegetation is an important one. In studying the effects of two different systems of grazing upon the same kind of vegetation in adjacent range pastures it became necessary to adopt a standard size of quadrat.

A number of factors influenced the determination. One of the most important of these was the method of comparing the vegetation in the pastures. It was decided to use a rather large number of list quadrats distributed fairly uniformly even though at random over the areas compared. This method would yield data on the abundance and frequency of all species including the important forage plants and overgrazing indicators. To save time it was desirable to use as small areas as possible. The time factor was important because the period during which the early spring plants are still green and the late summer plants tall enough to be identified is rather short and also because of the expense involved. Other factors that influenced the size of the quadrat were the kind, density, and uniformity of the vegetation.

The two pastures under study are located on the plain at the base of the foothills at an elevation of about 5,000 feet near Fort Collins, Colo. The physical conditions and vegetation in these pastures have been briefly described by Hanson and Ball (*6*).[4] In one pasture the deferred and rotation method of grazing had been practiced since 1921, in the other the continuous grazing method was used. (Fig. 1.) By the deferred and rotation method grazing was deferred for two years in succession on one half of the pasture until about August 15; on the other half meanwhile grazing was permitted from the time the range was ready in the spring, about May 1 to 7. At the end of two years the portion which had been grazed early was allowed to rest until about August 15 while the other half was grazed. Only adjacent portions of the pastures that were similar in relief and soil were studied in detail. Important differences in the vegetation, then, would appear to be due to the differences in the systems of grazing. The portion in the deferred and rotation pasture comprised about 72 acres and the portion in the other pasture comprised about 63 acres.

[1] Received for publication June 17, 1930; issued October, 1930.

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[3] The authors acknowledge the assistance given them by Andrew Clark, associate professor of mathematics, Colorado Agricultural College, who suggested the statistical methods that were used to analyze the data on abundance and frequency.

[4] Reference is made by number (italic) to Literature Cited. p. 560.

METHODS

The method employed to locate the quadrats was to lay out three lines equidistant from each other and parallel with the width of the pasture. One of these lines was near the middle of the area under study, the other two toward the ends. On each of these lines 10 points for quadrat studies were located at intervals of 125 feet (38 meters) in the deferred and rotation pasture and at intervals of 80 feet (24 meters) in the other.

The following sizes of quadrats were used at each of the 10 points on each line: 0.25, 0.5, 1, 2, 3, and 4 square meters. The area of 4 square meters included all of the other units. The total number of quadrats of each size in each pasture was 30. For each quadrat the species of plants were listed and tabulated. Whenever possible the individual stalks, as in Agropyron, Psoralea, and Artemisia, were counted. In the case of sod formers, such as Bouteloua and Bulbilis, descriptive terms were used to denote the abundance. The frequency

FIGURE 1.—View in the continuously grazed pasture showing the type of vegetation. *Agropyron smithii* and *Senecio perplexus* are prominent. May 21, 1929

and abundance of each species for each size of quadrat were determined (Tables 1 and 2) and the species were grouped on the basis of grazing value for cattle into three classes—desirable, undesirable, and immaterial. The desirable species are those that furnish considerable forage or that are highly palatable but because of their scarcity furnish little feed. The undesirable species are those that are poisonous, mechanically injurious, or that are not grazed and in addition make heavy demands upon essential factors such as soil moisture. Often they are strong and aggressive competitors. The immaterial species are those that are grazed but slightly, and often perhaps accidentally with other plants, or those that are not grazed and, moreover, appear not to make heavy demands upon the habitat, nor do they appear to be aggressive competitors.

TABLE 1.—*Frequency and abundance of species occurring in 30 quadrats of six different sizes in the deferred and rotation pasture, June 11–28, 1929* [a]

[The total figures of each size of quadrat are included in the next larger size]

Species	0.25 square meter quadrat		0.5 square meter quadrat		1 square meter quadrat		2 square meter quadrat		3 square meter quadrat		4 square meter quadrat	
	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance
Desirable species:	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*
Agropyron Smithii Rydb.	97	4, 210	100	8, 120	100	16, 281	100	27, 460	100	41, 762	100	53, 884
Schedonnardus paniculatus (Nutt.) Trel.	67	S	73	S	83	S	90	I	90	I	90	I
Senecio perplexus A. Nels.	47	81	57	161	63	335	70	591	77	875	80	1, 148
Eurotia lanata (Pursh) Moq.	27	16	50	29	60	46	70	69	73	98	73	124
Astragalus drummondii Dougl.	23	21	30	31	40	45	60	113	67	163	70	197
Bouteloua gracilis (H. B. K.) Lag.	27	S	33	S	47	I	47	I	57	I	57	I
Aristida longiseta Steud.	30	S	30	S	30	S	37	S	43	S	47	S
Helianthus pumilus Nutt.	10	6	17	10	27	29	30	51	37	66	47	87
Stipa viridula Trin.	13	13	17	25	17	53	20	110	20	168	20	229
Vicia linearis (Nutt.) Greene	3	5	3	7	3	14	3	23	3	38	3	50
Carex stenophylla Wahl.	0	0	0	0	0	0	0	0	0	0	0	0
Trifolium repens L.	0	0	0	0	0	0	0	0	0	0	0	0
Poa sp.	0	0	0	0	0	0	0	0	0	0	0	0
Undesirable species:												
Gutierrezia longifolia Greene	37	14	50	25	57	42	77	75	80	100	87	132
Iva axillaris Pursh	43	99	53	175	63	370	67	716	73	1, 023	73	1, 381
Psoralea tenuiflora Pursh.	47	28	50	48	57	94	63	165	70	220	73	283
Linum Lewisii Pursh	13	12	13	15	30	70	47	103	47	149	50	186
Artemisia gnaphalodes Nutt.	33	34	40	69	40	122	43	278	50	503	50	608
Bahia oppositifolia Nutt.	17	15	30	29	33	58	40	138	47	193	53	331
Bulbilis dactyloides (Nutt.) Raf.	13	V. S.	17	V. S.	23	S	37	I	43	I	53	I
Helianthus annuus L.	3	1	17	8	23	14	33	31	43	57	47	72
Opuntia humifusa Raf.	0	0	7	1	13	4	23	8	27	13	37	19
Quincula lobata (Torr.) Raf.	7	4	10	10	10	12	20	22	23	52	23	81
Muhlenbergia gracillima Torr.	13	V. S.	13	V. S.	17	S	17	S	20	S	20	S
Yucca glauca Nutt.	0	0	7	2	7	2	17	9	20	10	20	13
Artemisia frigida Willd.	3	4	3	4	7	5	17	12	23	14	30	19
Aster hebecladus DC.	7	19	10	58	10	104	13	282	13	365	17	513
Artemisia dracunculoides Pursh.	0	0	7	5	10	8	13	17	13	19	20	28
Argemone intermedia Sweet	0	0	3	1	10	9	10	9	10	9	10	10
Sitanion brevifolium J. G. Smith.	3	12	7	19	7	19	10	22	13	25	17	30
Hordeum nodosum L.	0	0	0	0	3	1	7	2	7	3	7	3
Cryptantha crassisepala (T. and G.) Greene	3	3	3	3	7	6	7	10	10	14	10	21
Grindelia squarrosa (Pursh) Dunal	0	0	0	0	3	1	3	1	3	3	3	3
Nothocalais cuspidata (Pursh) Greene	0	0	0	0	0	0	0	0	0	0	0	0
Bromus tectorum L.	0	0	0	0	0	0	0	0	0	0	0	0
Asclepias pumila (Gray) Vail	0	0	0	0	0	0	0	0	0	0	0	0
Euphorbia marginata Pursh.	0	0	0	0	0	0	0	0	0	0	0	0
Astragalus bisulcatus (Hook) Gray	0	0	0	0	0	0	0	0	0	0	0	0
Sporobolus cryptandrus (Torr.) Gray	0	0	0	0	0	0	0	0	0	0	0	0
Stanleya pinnata (Pursh) Brit.	7	2	7	5	7	5	7	9	10	12	10	16
Carduus undulatus Nutt.	0	0	0	0	0	0	0	0	0	0	0	0
Mamillaria missouriensis Sweet	0	0	0	0	0	0	0	0	0	0	3	1
Stipa vaseyi Scribn.	0	0	0	0	0	0	0	0	0	0	0	0

[a] The following abbreviations are used in Tables 1 and 2: V. S., very scarce; S, scarce; I, infrequent; F. frequent; Ab, abundant.

TABLE 1.—*Frequency and abundance of species occurring in 30 quadrats of six different sizes in the deferred and rotation pasture, June 11–28, 1929*—Continued

Species	0.25 square meter quadrat		0.5 square meter quadrat		1 square meter quadrat		2 square meter quadrat		3 square meter quadrat		4 square meter quadrat	
	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance
	Per cent	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*
Immaterial species:												
Sophora sericea Nutt.	70	73	97	139	97	264	100	466	100	712	100	938
Gaura coccinea Nutt.	50	53	63	83	90	197	97	342	97	584	97	727
Musineon divaricatum (Pursh) C. and R.	90	163	97	291	97	617	97	1,010	100	1,573	100	1,928
Malvastrum coccineum (Pursh) Gray	63	90	77	161	77	291	83	537	93	731	93	927
Draba nemorosa L.	43	142	63	360	70	637	80	956	80	1,393	80	1,566
Sophia pinnata (Walt.) Brit.	23	13	43	30	63	74	70	131	80	216	90	261
Allium reticulatum Fraser	10	4	20	5	40	28	67	67	73	108	80	121
Viola nuttallii Pursh	27	10	37	18	57	38	63	67	80	105	83	129
Polygonum douglasii Greene	30	19	37	32	47	49	60	73	67	106	67	154
Lepidium apetalum Willd.	40	41	43	74	47	119	50	194	60	312	63	426
Lappula occidentalis (Wats.) Greene	23	17	37	43	50	89	50	126	60	168	60	214
Astragalus hypoglottis L.	23	47	23	100	37	181	43	246	53	395	60	547
Lithospermum angustifolium Michx.	7	5	27	11	37	18	40	34	47	48	47	48
Astragalus shortianus Nutt.	0	0	10	3	23	8	37	15	40	26	43	32
Lactuca pulchella DC.	3	1	17	6	23	8	33	20	40	28	53	38
Evolvulus pilosus Nutt.	17	21	23	40	30	71	33	110	40	160	47	258
Allionia linearis Pursh	0	0	0	0	17	9	27	17	40	28	47	32
Petalostemon oligophyllus (Torr.) Rydb.	10	10	13	17	20	40	27	59	30	95	33	120
Lesquerella montana (Gray) Wats.	3	1	13	4	20	10	20	16	23	19	30	25
Plantago purshii R. and S.	10	12	17	26	17	40	17	108	20	145	20	187
Taraxacum officinale Weber	7	2	13	10	17	12	17	14	20	17	23	19
Eriogonum effusum Nutt.	7	2	10	3	10	5	17	18	20	24	30	30
Androsace occidentalis Pursh	3	1	13	14	13	15	13	22	17	37	17	41
Liatris punctata Hook.	7	3	7	3	10	16	13	22	30	27	33	41
Astragalus flexuosus (Dougl.) Hook.	3	7	7	22	13	51	13	64	17	93	20	118
Astragalus missouriensis Nutt.	7	3	7	7	7	13	13	23	13	32	17	51
Sideranthus spinulosus (Pursh) Sweet	7	3	7	4	10	5	13	12	13	13	13	15
Euphorbia robusta (Engelm.) Small	3	1	3	1	13	14	13	25	13	27	17	35
Festuca octoflora Walt.	10	47	10	73	10	114	10	232	17	352	17	418
Leucocrinum montanum Nutt.	0	0	0	0	3	1	10	4	10	5	10	5
Pentstemon secundiflorus Benth.	3	1	3	1	3	3	7	4	7	8	7	9
Hedeoma hispida Pursh.	0	0	3	1	7	2	7	2	7	2	7	4
Astragalus tridactylicus Gray	0	0	0	0	0	0	3	6	3	6	3	6
Specularia leptocarpa (Nutt.) Gray	0	0	3	1	3	1	3	1	7	2	7	2
Aragallus albiflorus A. Nels.	0	0	0	0	3	1	3	1	3	1	10	3
Cogswellia orientalis Jones	0	0	0	0	0	0	3	1	10	5	13	7
Pentstemon angustifolius Pursh	0	0	0	0	3	1	3	1	3	1	7	2
Tragopogon pratensis L.	0	0	0	0	0	0	3	1	3	1	7	2
Collomia micrantha Kell.	0	0	0	0	3	1	3	1	3	1	3	1
Malacothrix sonchoides (Nutt.) T. and G.	3	3	3	3	3	3	3	3	3	3	3	3
Lygodesmia juncea Don.	0	0	0	0	0	0	0	0	0	0	0	0
Myosurus minimus L.	3	1	3	1	3	11	3	11	3	13	3	13
Chenopodium album L.	0	0	3	1	3	2	3	2	3	2	3	4
Paronychia jamesii T. and G.	0	0	0	0	0	0	3	2	3	2	3	2
Comandra pallida A. DC.	3	2	3	4	3	4	3	4	3	8	3	14
Thelesperma gracile (Torr.) Gray	0	0	0	0	0	0	0	0	0	0	0	0

TABLE 1.—*Frequency and abundance of species occurring in 30 quadrats of six different sizes in the deferred and rotation pasture, June 11–28, 1929*—Contd.

Species	0.25 square meter quadrat		0.5 square meter quadrat		1 square meter quadrat		2 square meter quadrat		3 square meter quadrat		4 square meter quadrat	
	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance
Immaterial species—Contd.	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*
Camelina sativa Crantz	0	0	0	0	0	0	0	0	0	0	3	3
Townsendia exscapa (Rich.) Por	0	0	0	0	0	0	0	0	3	1	3	1
Eriocoma cuspidata Nutt.	0	0	0	0	0	0	0	0	3	1	3	1
Erysimum asperrimum (Greene) Rydb	0	0	0	0	0	0	0	0	3	2	3	2
Scutellaria brittonii Porter	0	0	0	0	0	0	0	0	3	3	7	10

TABLE 2.—*Frequency and abundance of species occurring in 30 quadrats of six different sizes in the continuously grazed pasture, June 11–28, 1929*

Species	0.25 square meter quadrat		0.5 square meter quadrat		1 square meter quadrat		2 square meter quadrat		3 square meter quadrat		4 square meter quadrat	
	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance
Desirable species:	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*
Agropyron smithii	100	2,284	100	4,777	100	9,807	100	17,921	100	27,810	100	36,942
Schedonnardus paniculatus	30	V. S.	43	V. S.	57	8	60	8	67	8	70	8 2
Senecio perplexus	13	8	23	22	23	43	40	85	40	136	40	170
Eurotia lanata	0	0	0	0	0	0	0	0	0	0	0	
Astragalus drummondii	10	15	13	17	20	40	30	61	37	122	37	147
Bouteloua gracilis	67	F	73	F	73	F	73	F	83	Ab	87	Ab
Aristida longiseta	43	S	50	S	57	S	64	S	67	S	77	F
Helianthus pumilus	13	13	20	18	37	55	40	91	57	147	60	214
Stipa viridula	0	0	0	0	0	0	0	0	3	4	3	4
Vicia linearis	0	0	0	0	3	2	3	7	3	9	3	16
Carex stenophylla	0	0	0	0	0	0	3	241	3	560	3	828
Trifolium repens	0	0	0	0	3	1	3	1	3	1	3	1
Poa sp	0	0	0	0	0	0	3	V. S.	3	V. S.	3	V. S.
Undesirable species:												
Gutierrezia longifolia	23	8	37	15	53	25	73	48	83	68	87	86
Iva axillaris	37	97	37	180	43	370	43	606	47	821	50	1,021
Psoralea tenuiflora	60	75	73	131	90	286	100	544	100	825	100	1,099
Linum lewisii	17	13	20	31	30	62	33	107	37	135	40	159
Artemisia gnaphalodes	27	32	30	67	33	130	53	260	60	374	60	579
Bahia appositifolia	20	57	23	105	33	166	37	275	60	392	60	412
Bulbilis dactyloides	23	S	23	S	23	S	23	S	37	S	37	S
Helianthus annuus	0	0	0	0	3	1	10	3	10	6	13	9
Opuntia humifusa	0	0	0	0	0	0	13	3	23	7	37	14
Quincula lobata	0	0	0	0	0	0	7	3	10	4	10	4
Muhlenbergia gracillima	3	V. S.	3	V. S.	3	V. S.	3	V. S.	3	V. S.	3	V. S.
Yucca glauca	0	0	0	0	7	2	20	8	23	13	27	1
Artemisia frigida	17	6	17	18	27	32	47	57	53	81	57	107
Aster hebecladus	17	63	17	91	23	121	37	193	40	240	43	397
Artemisia dracunculoides	0	0	10	3	20	6	27	9	30	13	37	16
Argemone intermedia	0	0	0	0	17	9	17	9	17	9	20	15
Sitanion brevifolium	7	3	13	9	17	17	27	34	37	48	40	41
Hordeum nodosum	0	0	0	0	3	2	10	20	10	20	10	29
Cryptantha crassisepala	0	0	0	0	0	0	0	0	0	0	0	2
Grindelia squarrosa	3	1	3	1	7	2	13	4	17	5	17	0
Nothocalais cuspidata	7	2	7	2	7	3	10	9	10	9	10	16
Bromus tectorum	7	7	7	7	7	10	17	35	23	75	27	90
Asclepias pumila	3	8	3	5	3	19	3	30	3	30	3	48
Euphorbia marginata	3	1	3	1	3	1	3	1	3	1	3	2
Astragalus bisulcatus	0	0	0	0	3	12	3	12	3	13	3	11
Sporobolus cryptandrus	3	V. S.	3	V. S.	3	V. S.	3	V. S.	3	V. S.	3	V. S3

TABLE 2.—*Frequency and abundance of species occurring in 30 quadrats of six different sizes in the continuously grazed pasture, June 11–28, 1929*—Continued

Species	0.25 square meter quadrat		0.5 square meter quadrat		1 square meter quadrat		2 square meter quadrat		3 square meter quadrat		4 square meter quadrat	
	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance	Frequency	Abundance
	Per cent	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*	*Per cent*	*Number*
Undesirable species—Contd.												
Stipa vaseyi	0	0	0	0	0	0	0	0	3	1	3	1
Carduus undulatus	3	1	3	2	3	2	3	2	3	2	3	2
Stanleya pinnata	0	0	0	0	0	0	0	0	0	0	3	1
Immaterial species:												
Sophora sericea	30	32	33	50	40	92	43	173	47	224	47	304
Gaura coccinea	43	28	57	53	80	115	93	194	97	297	97	398
Musineon divaricatum	87	121	90	230	93	405	97	771	100	1,204	100	1,546
Malvastrum coccineum	47	45	70	86	77	181	83	339	87	495	90	627
Draba nemorosa	37	167	47	282	57	554	63	958	67	1,454	71	1,774
Sophia pinnata	33	25	43	50	70	116	83	211	93	269	97	343
Allium reticulatum	7	4	10	6	10	6	30	16	30	18	33	25
Viola nuttallii	7	2	13	5	23	13	30	18	33	27	43	34
Polygonum douglasii	3	1	13	8	13	9	13	12	17	13	17	14
Lepidium apetalum	17	13	37	23	40	43	53	78	60	110	60	127
Lappula occidentalis	13	6	17	10	20	39	23	58	33	78	40	93
Astragalus hypoglottis	20	36	30	65	43	124	47	303	57	509	67	634
Lithospermum angustifolium	0	0	3	1	7	2	7	2	17	12	20	18
Astragalus shortianus	3	1	3	1	10	4	17	13	20	17	23	20
Lactuca pulchella	0	0	0	0	3	2	27	12	40	17	43	25
Evolvulus pilosus	17	24	23	34	33	89	50	194	60	300	67	372
Allionia linearis	13	6	13	7	20	11	33	24	43	35	50	43
Petalostemon oligophyllus	3	1	7	2	13	7	20	20	33	58	40	69
Lesquerella montana	3	1	7	2	7	6	10	11	20	18	27	27
Plantago purshii	10	6	17	10	20	19	23	38	33	73	37	91
Taraxacum officinale	0	0	3	1	3	1	7	3	10	5	13	7
Eriogonum effusum	3	1	7	2	20	9	27	15	37	31	40	38
Androsace occidentalis	3	1	3	3	7	10	7	10	7	40	7	40
Liatris punctata	13	6	27	26	37	54	43	89	57	161	63	217
Astragalus flexuosus	20	22	27	27	27	60	33	104	40	188	43	222
Astragalus missouriensis	20	11	30	14	43	26	60	67	73	105	73	128
Sideranthus spinulosus	10	1	10	3	13	6	13	6	30	14	33	18
Euphorbia robusta	3	1	10	7	10	10	17	14	20	17	23	20
Festuca octoflora	13	6	17	28	17	83	20	141	23	217	27	250
Leucocrinum montanum	7	2	7	2	7	2	13	4	20	7	20	10
Pentstemon secundiflorus	0	0	0	0	0	0	0	0	0	0	0	0
Hedeoma hispida	0	0	0	0	0	0	3	1	3	3	3	9
Astragalus tridactylicus	3	1	3	1	3	1	3	1	7	5	10	15
Specularia leptocarpa	0	0	0	0	0	0	0	0	0	0	0	0
Aragallus albiflorus	0	0	0	0	0	0	0	0	0	0	3	1
Cogswellia orientalis	3	3	7	7	7	9	7	10	10	12	13	16
Pentstemon angustifolius	3	4	7	5	7	11	17	17	20	19	20	20
Tragopogon pratensis	3	1	3	1	7	2	10	3	17	5	23	7
Collomia micrantha	0	0	0	0	0	0	0	0	0	0	0	0
Malacothrix sonchoides	0	0	0	0	0	0	0	0	0	0	0	0
Lygodesmia juncea	17	12	17	15	20	29	30	58	40	94	40	115
Myosurus minimus	0	0	0	0	0	0	0	0	0	0	0	0
Chenopodium album	0	0	0	0	0	0	0	0	0	0	0	0
Paronychia jamesii	0	0	0	0	0	0	3	1	3	1	3	1
Comandra pallida	3	2	3	2	3	8	3	9	3	14	3	15
Thelesperma gracile	0	0	3	2	3	2	3	2	7	10	7	12

ANALYSIS OF THE DATA

In attempting to decide which of the six sizes of quadrats was the best to use, four methods of analysis were employed. The first of these was to compare the total number of species found in each size. The second was to analyze the data on abundance and the third and fourth were frequency methods.

TOTAL NUMBER OF SPECIES

The total number of species found in the quadrats in the deferred and rotation pasture ranged from 56 in the smallest size to 81 in the largest size. In the continuously grazed pasture the number of species

ranged from 58 to 80. The total number of species that have been found in the areas of both pastures is 109, and only 22 species are not recorded from any of the quadrats. Usually the individuals of these latter species are very scarce and often decidedly local in distribution. Graphs showing these variations are presented in Figure 2.

The curves representing the total number of species in the quadrats are very similar for the two pastures. Both of them show a decided tendency to flatten between the 1 and 2 square meter sizes. This flattening continues as the size of the quadrat increases. Braun-Blanquet (*1*) shows that in analyzing vegetation the size of the minimum area for quadrats should be where the curve begins to become horizontal.

The graph, then, indicates that the minimum size of the quadrat should not be smaller than 1 square meter, that 2 square meters is

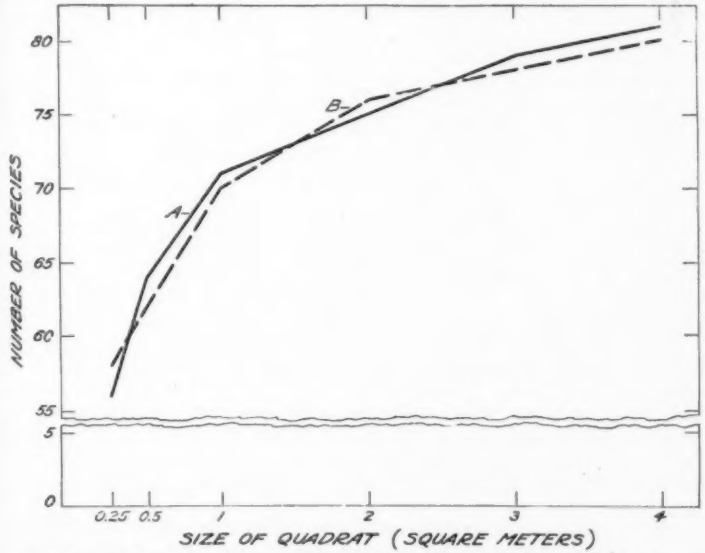

FIGURE 2.—Number of species in a total of 30 quadrats according to the size of the quadrat: A, Continuous line, represents the number of species in the deferred and rotation pasture; B, broken line, the number in the continuously grazed pasture

somewhat better, and that the benefits to be gained by using still larger sizes hardly warrant the extra time that would be required. The few additional species found in the quadrats above the 2 square meter size are so sparsely represented that they appear to be of very minor importance from the plant sociological standpoint and of no importance in range management.

ABUNDANCE

The total number of stalks of all species except sod formers or bunch grasses was secured for each size of quadrat for each pasture. Ratios were then obtained between the same size quadrats in the two pastures. In this way six comparative ratios were secured as shown in Table 3.

TABLE 3.—*Total number of stalks of all species in each pasture, 30 quadrats of each size being represented*

Pasture	0.25 square meter	0.5 square meter	1 square meter	2 square meters	3 square meters	4 square meters
Deferred and rotation	5,397	10,452	20,812	35,388	53,597	69,009
Continuous	3,287	6,583	13,386	24,679	38,157	50,245
Ratio	1.642	1.588	1.556	1.434	1.403	1.374

The average of the six ratios given in Table 3 is 1.4995 ± 0.01974. Since this average is between those of the 1 and 2 square meter sizes it indicates that, of the sizes employed, the most desirable quadrat to use in comparing the vegetation in the two pastures lies between 1 and 2 square meters. The average ratios in each of the three separate groups of desirable, undesirable, and immaterial species also point to a size between the 1 and 2 square meter size.

FREQUENCY

The percentages of frequency for each species in Tables 1 and 2 were secured by dividing the number of quadrats in which the species was found by the total number of quadrats. For example, if a species occurred in 15 quadrats in one pasture the percentage of frequency would be secured by dividing 15 by 30 (the total number of quadrats in each pasture), giving 50 per cent.

In order to find out whether the data on frequency might indicate the use of one size of quadrat in preference to other sizes, average weighted ratios were secured (Burgess, *2, p. 16–40*). For each species and for each size of quadrat the number of quadrats in which the species occurred was divided by the number of quadrats in which it occurred in the next larger size. This ratio expresses the relationship between the frequency in the two sizes of quadrats. This ratio was then weighted by multiplying it by the number of quadrats in which the species occurred in the larger of the two sizes. For example, in the continuously grazed pasture *Agropyron smithii* occurs in 30 quadrats in each of the sizes of quadrats. Therefore, 30 (in 0.25 square meter size) divided by 30 (in 0.5 square meter size) equals 1, which is the ratio of relationship. In order to weight this ratio it was multiplied by 30 (the number of quadrats in which it occurred in 0.5 square meter size), giving 30. In the case of Schedonnardus it is 9 (in 0.25 square meter size) divided by 13 (in 0.5 square meter size) which equals 0.69, and this ratio multiplied by 13 (in 0.5 square meter size) equals 8.97. This was done for each species in each size of quadrat.

The weighted ratios in each size of quadrat were added. The number of quadrats in which each species occurred in the next larger size of quadrat was also added. Then the former was divided by the latter to give the weighted average ratio expressing relationship of frequency of each size of quadrat to the next larger size. This method of computation may be expressed in the following formula

$$R = \frac{\Sigma fx}{\Sigma f},$$

where

R = weighted average ratio.
Σ = summation.

f = number of quadrats in which each species occurred in the larger of the two quadrats.

χ = the ratio of relationship of each species of a certain size in which each species occurred divided by the number of quadrats of the next larger size in which it occurred.

The results of this method of computation are given in Table 4.

TABLE 4.—*Weighted average ratios of relationship in frequency of each size of quadrat to the next larger size*

Pasture	0.25 square meter	0.5 square meter	1 square meter	2 square meters	3 square meters	Average
Deferred and rotation	0.767	0.799	0.839	0.882	0.924	0.842±0.01545
Continuous	.790	.787	.796	.837	.935	.829± .01608

Table 4 shows that the average ratios are 0.842 ± 0.01545 and 0.829 ± 0.01608. Since these averages are between the 1 and 2 square meter sizes the conclusion may be drawn that the size to use in comparing the vegetation should not be smaller than 1 square meter. If smaller sizes were used, then it appears that the area would not be a fair representation of the vegetation. In order for a species to be "constant" it appears that it should be present in 84 per cent or more of the quadrats in the deferred and rotation pasture and 82 per cent or more of those in the other pasture. This also points to the use of quadrats not smaller than 1 or 2 square meters.

Much work has been done in Europe on the minimum area to use in making quadrat studies in analyzing the vegetation. The term "minimum area" has been defined as the smallest area upon which the association reaches its definitive number of constants. Nichols (*8, p. 134*) states:

> In areas of progressively smaller size than this minimum area, the number of constants rapidly diminishes; in areas of larger size, up to a certain point, it remains constant.

The minimum areas reported by Du Rietz (*3, p. 423–427*) usually lie between 1 and 4 square meters when a frequency of 90 per cent or more is used for the determination of constants.

The constants in the deferred and rotation pasture, using a frequency of 84 per cent as the lower limit for constancy and a minimum area of 2 square meters, are *Agropyron smithii, Schedonnardus paniculatus, Sophora sericea, Gaura coccinea,* and *Musineon divaricatum.* The species in the continuously grazed pasture having a frequency above 82 per cent are Agropyron, Gaura, Musineon, *Malvastrum coccineum, Sophia pinnata,* and *Psoralea tenuiflora.* The last appears to be a good indicator of overgrazing in this area.

In larger sizes of quadrats the constants increase in number, and in smaller sizes they decrease. Since the number of constants does not reach a constant number in the sizes of quadrats used for minimum frequencies between 60 and 90 per cent it might be that all of these sizes are too small to use in determining the structure of the vegetation. But for the purpose of determining the effects of different systems of grazing upon the vegetation in the two pastures it appears that the 1 or 2 square meter size is large enough.

Raunkiaer's Law of Frequency

Since Kenoyer (*7*) has given an excellent presentation of this law it is unnecessary to describe it in detail. Raunkiaer has formulated five frequency classes. Species with frequencies between 1 and 20 per cent are in class A, 21 to 40 in class B, 41 to 60 in class C, 61 to 80 in class D, and 81 to 100 in class E. The size and number of the quadrats influence the number of species that occur in each class. Raunkiaer and Kenoyer used 25 quadrats, usually 0.1 square meter in area, for each type of vegetation. Gleason (*4*) prefers 100 quadrats ranging in size from 1 square meter for dense closed vegetation to 4 square meters for open or irregular communities. Both Kenoyer (*7*) and Gleason (*5*) state that the highest number of species is found in class A, that the number of species usually decreases in each class except E, which has more than class D. The curve expressing number of species in each class of frequency has two peaks, a high one in class A and a low one in class E. Gleason (*5, p. 407*) says that the frequency of a species is shown only when the quadrats are of such size that the resulting percentages of frequency cover the whole range of classes from A to E, and when of this size the double peak in classes A and E is often apparent. He states:

> Any increase in size tends to move the index of the various species into progressively lower classes and may completely remove the peak in class V [E]. Any increase in size tends to move them into progressively higher classes and thereby to accentuate the peak in class V, while the greater total area covered continues to add more rare species to Group I [A].

Table 5.—*Percentage of species in each class of frequency and total number of species for each size of quadrat in each pasture*

Size of quadrat, sq. meter	Class A, 1 to 20 per cent	Class B, 21 to 40 per cent	Class C, 41 to 60 per cent	Class D, 61 to 80 per cent	Class E, 81 to 100 per cent	Total number of species
DEFERRED AND ROTATION PASTURE						
0.25	61	21	9	5	4	56
.5	61	17	11	6	5	64
1.0	55	20	11	7	7	71
2.0	53	16	11	12	8	75
3.0	51	14	14	14	7	79
4.0	49	10	19	11	11	81
CONTINUOUS PASTURE						
0.25	74	14	7	2	3	58
.5	63	19	10	5	3	62
1.0	60	19	11	6	4	70
2.0	50	23	12	7	8	76
3.0	44	27	13	6	10	78
4.0	40	26	14	10	10	80

It appeared from these statements that a classification into frequency classes of the species in the range pastures under study might indicate the most suitable size to use. Therefore such a classification was made. The results are shown in Table 5. Analysis of this table shows that for each size of quadrat in both pastures the percentage of the total number of species is always greatest in class A and that the percentage in this class declines as the area becomes larger. In the deferred and rotation pasture the percentage of species usually becomes progressively lower as the frequency becomes higher and double peaks do not appear except in the 4 meter size, where the

second, smaller peak is in class C. The curves of all the sizes except the 4 meter size decline abruptly from class A to class B and then show a gradual tendency to become horizontal.

In the continuously grazed pasture, however, double peaks, a high one in class A and a very low one in class E, are evident in the 0.25, 2, and 3 square meter sizes. The other two sizes do not show the double peak. The curves differ from those of the other pasture in showing a less abrupt decline between the A and B classes and a slower tendency to become horizontal.

The analysis of the data, according to Raunkiaer's law, does not appear to indicate that there is any pronounced advantage in using any particular size of quadrat between 0.25 and 4 square meters. All of the sizes of quadrats give percentages in each class of frequency. The second peak, in class E, is not present in the data from the deferred and rotation pasture, and it appears in the 0.25, 2 and 3 square meter sizes in the other pasture. Perhaps still larger sizes of quadrats would have yielded a more pronounced tendency to produce peaks in class E but the extra expense entailed in using larger sizes would hardly be warranted.

SUMMARY AND CONCLUSIONS

In order to determine the most suitable size of quadrat to use in studying differences in the abundance and frequency of species in adjacent pastures grazed by two different methods, data were secured on six sizes of quadrats, ranging from 0.25 to 4 square meters. Thirty quadrats, distributed fairly uniformly and at random, were used for each size of quadrat in each pasture.

The first three methods that were used to analyze the data indicated that the size of the quadrat should not be smaller than 1 or 2 square meters. Curves showing the total number of species found in the 30 quadrats for each size of quadrat showed a decided flattening at the 1 square meter size. This continued in the 2 square meter size. The graph (fig. 2), then, indicates that the size of the quadrat should not be smaller than 1 square meter, that 2 square meters is somewhat better, but that the use of larger quadrats does not warrant the extra expense involved.

The second method of analyzing the data was to secure the ratios of the total number of stalks of plants in each size quadrat between the two pastures. Since the average of these six ratios was between those of the 1 and 2 square meter sizes it indicates that, of the sizes used, either one of these would be satisfactory.

The third method was to secure weighted average ratios of relationship in frequency of each size of quadrat to the next larger size for both pastures. Since the averages were again between those of the 1 and 2 square meter size it may be concluded that the quadrat should not comprise an area less than 1 square meter.

The fourth method, by which the data were analyzed according to Raunkiaer's law of frequency, failed to indicate any advantage in using any particular size of quadrat.

Because of the evidence secured by the first three methods and because the use of larger areas would not yield results commensurate with the additional expense, it appears that the most suitable size of quadrat to use in this type of vegetation is 2 square meters lying adjacent to each other.

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EFFECT OF CATTLE GRAZING ON VEGETATION OF A VIRGIN FOREST IN NORTHWESTERN PENNSYLVANIA [1]

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INTRODUCTION

The grazing studies carried out in forest stands usually have had as their object the determination of the effect of grazing animals on the more important tree species. Little if any attention has been paid to the effect of grazing animals on forest density and composition as a whole. This is particularly true of the studies made in forests in the eastern part of the United States.

The primary object of the present study was to determine the effect of grazing upon the composition and density of vegetation in a virgin forest. In addition, an attempt was made to learn the nature and extent of the injuries caused to various species of trees by the grazing animals. Observations were made on the effect of grazing animals upon certain soil conditions.

DESCRIPTION OF THE AREAS STUDIED

The study here reported was made during the summer of 1929 in a hemlock-beech stand (more specifically, a hemlock, beech, white-pine, and red-maple stand) at Heart's Content, Warren County, in northwestern Pennsylvania. For a detailed description of this stand the reader is referred to an earlier paper by the writer (*1*).[3] The conditions within this stand are essentially those of a virgin forest; only a relatively small amount of cutting has been done.

A portion of the area, about 25 acres, has been grazed and is inclosed by a barbed-wire fence. The adjacent forested area has not been grazed. There is every reason to believe that prior to grazing both areas supported the same kind of vegetation; relief and dominant trees on the two areas are essentially similar at present. Figure 1 shows the general conditions existing on the two areas. Adjacent to the grazed area, on the northwest side, there is a cleared field of about 5 acres which supports a grass sod. A small stream enters the grazed area along the south side and flows southward through the ungrazed area.

HISTORY OF THE GRAZED AREA

Grazing has been carried on more or less continuously for at least 20 years. It was reported by a local resident that in 1909, 5 cows were being pastured within the stand. For a period of 12 years prior

[1] Received for publication May 24, 1930; issued October, 1930. The field work on which this paper is based was done while the writer was associate silviculturist, Allegheny Forest Experiment Station, U. S. Department of Agriculture.

[2] The writer is indebted to H. P. Clark, formerly field assistant, Allegheny Forest Experiment Station, for field assistance in gathering the data which form the basis of this report. Acknowledgment is also made to Dr. J. P. Kelly, professor of botany, Pennsylvania State College, for aid in identifying certain grasses.

Reference is made by number (italic) to Literature Cited, p. 570.

to 1928, 12 cows grazed on the area; during 1929, only 2. For a period of about 2 years, some time between 1909 and 1928, no stock were on the area. Cattle are the only animals which have been grazed, and they used the area only as night pasture from about June to October each year. In addition to the 25 acres of forest land, the stock had access to the adjacent 5 acres of grassland. Utilization of the forage within the forested area appears to have been moderately close.

METHODS

Using the barbed-wire fence which separated the grazed from the ungrazed area as a base line, strips were run out in opposite direc-

FIGURE 1.—A barbed-wire fence separates the ungrazed area (right) from the grazed area (left). Note the absence of young tree growth on the grazed side

tions into the two areas. The strips were from 100 to 200 feet apart and were established with a hand compass and 50-foot steel tape. The length of the strips varied from 200 to 500 feet; at 50-foot intervals along each strip a square quadrat of 1 milacre (6.6 by 6.6 feet, or 0.001 acre) was established. No quadrats were established within 50 feet of the fence, nor in the cleared field adjacent to the forest. On each quadrat the following data were taken:

(1) Number of individuals of each tree species in the following size classes:

Size class	Height in feet
1	0 to 1.0
2	1.1 to 2.5
3	2.6 to 6.0
4	6.1 to 15.0
5	15.1 to 25.0

Trees above 25 feet in height were not recorded since they were not believed to be as subject to the grazing influence as the younger trees;

forest growth above 25 feet in height appeared to be essentially the same on both the grazed and ungrazed areas.

(2) List of shrubby and herbaceous plants.

(3) Degree of cover in each of the above five size classes. Five degrees of cover were recognized:

Degree of cover	Per cent cover
1	0 to 20
2	20.1 to 40
3	40.1 to 60
4	60.1 to 80
5	80.1 to 100

(4) Diameter at breast height of all trees 0.6 inch and over.

In addition to the above data, notes were taken as to the injuries suffered by the various tree species due to grazing; the general condition of the surface soil relative to structure and consistency; the depth of the organic layers and general state of their decomposition.

The nomenclature here adopted is that of Sudworth (*6*) for the trees, and that of Gray's Manual (*3*) for the shrubs and herbs.

EFFECT OF GRAZING ON TREE SPECIES

INJURIES

The direct injuries sustained by mature trees as a result of grazing are chiefly in the roots. The extent of such injury is dependent, to a large degree, on the depth of rooting of the particular species and on the class of stock grazed. Shallow-rooted species such as beech (*Fagus grandifolia*) and hemlock (*Tsuga canadensis*) may be severely injured by heavy animals, such as cattle. The root damage at Heart's Content was most apparent in the case of beech, since root-suckers usually appeared from the vicinity of each wound and thus marked the injuries. In hemlock, the damage was not as apparent, since this species does not produce root suckers; however, there is no reason to believe that the damage to hemlock was any less severe than to beech. Besides occasionally killing the smaller roots, the injuries also open the way for fungus infection.

Browsing of young tree growth is selective and seems to be largely governed by the palatability of the various species. As a rule, damage to hardwoods is greater than damage to conifers. This fact was clearly brought out in the present study. The reduction in number of individuals per acre in size classes 2, 3, 4, and 5 which may be attributed to grazing is as follows for the three most common species: Hemlock 61 per cent, red maple (*Acer rubrum*) 81 per cent, and beech 85 per cent. That coniferous trees are not immune to browsing damage is further shown by the fact that on the grazed area about 35 per cent of the quadrats showed damage to the hemlock. A group of young hemlock trees which have been severely browsed is shown in Figure 2. Ninman and Thompson (*2*) in studying a grazed area in Wisconsin, found that 26.8 per cent of the white pine (*Pinus strobus*) under 20 years of age had been injured by cattle. Probably hemlock is less susceptible to browsing injury than some other coniferous species because of the relatively tough, flexible nature of its leader and branches.

The height of the young trees is also a factor in determining the extent and severity of damage by browsing and rubbing. In hemlock most of the browsing was confined to trees below 5 feet in height. Stickel and Hawley (*5*) state that in plantations, trees 3 feet and over in height are safe under carefully controlled grazing. Ninman and Thompson (*2*) found that injuries to branches of white pine were most common on trees between 3 and 7 feet in height. Sampson (*4*) states that aspen (*Populus tremuloides*) reproduction is no longer in danger of serious damage when the terminal shoot and some of the lateral branches have attained a height beyond which stock generally

FIGURE 2.—Young hemlock 2 to 4 feet in height severely browsed by cattle

browse. He found that in the case of cattle browsing the greatest damage was done to trees under 2½ feet in height.

Hardwoods appear to suffer most severely when they are small, between 2 and 12 inches in height, and damage continues until they are 7 to 8 feet in height. Hardwoods in general are more susceptible to browsing in both smaller and larger sizes than hemlock. Damage due to rubbing by the animals is most severe when the trees are 2 to 4 inches d. b. h. (diameter at breast height), and is practically negligible in trees over 6 inches d. b. h. A few instances were noted where the stock had actually pulled hemlock trees 12 inches high out of the ground. Damage due to pulling seedlings out is most apt to occur with shallow-rooted species, or with species on shallow or excessively moist soils.

In comparison with the number of the same species dead on the ungrazed area, the dead hemlock on the grazed area was about 113 per cent and beech 134 per cent. The increased number of dead trees

of these two species on the grazed area probably is due to the influence of the grazing animals. The reason for the relatively large number of dead red maple trees on the ungrazed area and their absence on the grazed area is not apparent. Comparative numbers of dead trees found are not considered as reliable an indication of the extent of grazing damage as are comparative numbers of live trees. Because of the trampling by animals, dead trees undoubtedly disappear more quickly on grazed areas than on areas not subject to grazing.

COMPOSITION

As shown in Table 1, the composition on the ungrazed and grazed areas is practically the same so far as number of species is concerned. In the five size classes studied there are 12 species on the ungrazed area and 15 on the grazed area. Yellow birch (*Betula lutea*), hawthorn (*Crataegus* sp.), black cherry (*Prunus serotina*), and large-leaved holly (*Ilex montana*) are present on the grazed area but absent on the ungrazed area. Sugar maple (*Acer saccharum*) is present on the ungrazed area but absent on the grazed area.

TABLE 1.—*Abundance and frequency of tree species on the ungrazed and grazed areas*

UNGRAZED AREA [a]

Species	Abundance (average number per acre)					Total	Frequency index [b] (per cent)					Dead (number per acre)
	Forest layer—						Forest layer—					
	1	2	3	4	5		1	2	3	4	5	
Pinus strobus	116					116	5					
Tsuga canadensis	4,533	967	567	450	50	6,567	70	25	25	25		167
Betula lenta				17	33	50					5	
Fagus grandifolia	666	217	367	633	250	2,133	15	15	20	40	15	83
Castanea dentata			33	17		50			5			17
Quercus borealis maxima	33			17		50	5					
Magnolia acuminata	67	33	17	17		134	5					
Hamamelis virginiana	83					83						
Amelanchier canadensis		17		17		34						
Prunus pennsylvanica	33					33	5					
Acer saccharum	17					17						
Acer rubrum	2,550	283	67	200	17	3,117	60	15	5	15		83

GRAZED AREA [c]

Pinus strobus	95					95	10					
Tsuga canadensis	5,603	381	174	190	47	6,395	75	15	10	10	5	190
Betula lenta	80	16				96	5					
Betula lutea	16					16						
Fagus grandifolia	8,381	111		95	16	8,603	50	5		5		111
Castanea dentata	32					32	5					16
Quercus borealis maxima	47					47	5					
Magnolia acuminata	63					63	5					
Hamamelis virginiana	16	111	47			174						
Amelanchier canadensis	32					32	5					
Crataegus sp.	47					47						
Prunus serotina	63					63	5					
Prunus pennsylvanica	32					32	5					
Ilex montana		16				16						
Acer rubrum	4,000	32		63	16	4,111	80			5		

[a] Based on 60-milacre plots. [b] Expressed in class intervals of 5. [c] Based on 63-milacre plots.

The abundance and frequency values of the three most common species, hemlock, beech, and red maple, are decidedly different on the two areas. In each case the abundance of these species in size class 1 was much higher on the grazed than on the ungrazed area. The

increase in numbers was greatest in the case of beech. On the grazed area the number of beech trees in size class 1 is about 1,258 per cent of the number in the same size class on the ungrazed area. The large amount of beech in size class 1 on the grazed area is practically all of root-sucker origin and most of it is only 1 or 2 inches high. It has been repeatedly browsed off by cattle. Even if protection were afforded it seems doubtful that the suckers would grow into good forest trees. The increased numbers of hemlock and red maple trees in size class 1 on the grazed area may be due to more favorable seedbed conditions and increased light.

In size classes 2, 3, 4, and 5 on the grazed area the abundance and frequency values of hemlock, beech, and red maple are decidedly lower than on the ungrazed area. (Table 1.) Red maple, particularly, appears to be almost entirely eliminated on the grazed area. It seems clear that continued grazing prevents full reproduction of the tree species, and in time will result in decreased density of the main crown canopy. Likewise it appears that the resultant forest stand will contain a relatively higher percentage of hemlock than the original forest.

BASAL AREA

Table 2 shows the basal areas of the young trees on the grazed and ungrazed plots. The basal-area values given represent trees 1 inch and over in diameter at breast height in size classes 3, 4, and 5. The basal area on the grazed side is about 41 per cent of that on the ungrazed side.

TABLE 2.—*Basal area of young trees on ungrazed and grazed areas*

Species	Basal area on ungrazed area [a]	Basal area on grazed area [a]	Species	Basal area on ungrazed area [a]	Basal area on grazed area [a]
	Sq. ft. per acre	*Sq. ft. per acre*		*Sq. ft. per acre*	*Sq. ft. per acre*
Tsuga canadensis	1.86	4.11	Hamamelis virginiana	.10	
Betula lenta	.73		Acer rubrum	3.86	.73
Fagus grandifolia	12.51	3.00			
Quercus borealis maxima	.10		Total	19.26	7.84
Magnolia acuminata	.10				

[a] Represented by trees 1 inch or more d. b. h., occurring in size classes 3, 4, and 5.

EFFECT OF GRAZING ON LESSER VEGETATION

COMPOSITION

Table 3 lists all the shrubby and herbaceous species found on the two areas, together with their frequency values in terms of percentage. Of the total of 70 species recorded, 27 are common to both areas. Nine species were found on the ungrazed area only and 34 species were found only on the grazed area. The following species of relatively high frequency are confined largely or wholly to the ungrazed area: *Lycopodium lucidulum*, *Coptis trifolia*, and *Viburnum alnifolium*. Apparently the conditions resulting from the presence of grazing animals inhibit the development of these species on the grazed area.

TABLE 3.—*Frequency of shrubby and herbaceous species on the ungrazed and grazed areas*

[Based on 60 ungrazed plots; 63 grazed]

Scientific name	Common name	Frequency (per cent)	
		Ungrazed area	Grazed area
Asipidium noveboracense	New York fern		8
Aspidium spinulosum	Common wood fern	78	88
Dicksonia punctilobula	Hay-scented fern		22
Onoclea sensibilis	Sensitive fern		2
Osmunda claytoniana	Interrupted fern	2	(a)
Osmunda cinnamomea	Cinnamon fern	7	
Lycopodium lucidulum	Shining club moss	22	2
Anthoxanthum odoratum	Sweet vernal grass		3
Brachyelytrum erectum	Bearded short-husk	2	
Phleum pratense	Timothy		(a)
Agrostis alba	White bent grass		(a)
Poa sp	Meadow grass		(a)
Juncus tenuis	Slender rush		2
Oakesia sessilifolia	Sessile-leaved bellwort	7	10
Clintonia umbellulata	Speckled clintonia	3	
Maianthemum canadense	Wild lily-of-the-valley	43	50
Streptopus roseus	Rosy twistedstalk	2	(a)
Medeola virginiana	Cucumber root	3	3
Trillium erectum	Wake-robin	3	3
Habenaria orbiculata	Roundleaf orchis	2	(a)
Corallorrhiza maculata	Coralroot	2	
Laportea canadensis	Wood nettle		2
Pilea pumila	Richweed	2	18
Rumex obtusifolius	Bitter dock		14
Rumex acetosella	Field sorrel	2	3
Polygonum hydropiper	Common smartweed	3	21
Phytolacca decandra	Common pokeberry		2
Stellaria media	Common chickweed		(a)
Ranunculus acris	Tall buttercup		6
Coptis trifolia	Gold thread	20	
Podophyllum peltatum	Mayapple		6
Cardamine pennsylvanica	Bittercress		(a)
Fragaria virginiana	Strawberry		2
Potentilla recta	Rough-fruited cinquefoil		3
Potentilla canadensis	Fivefinger		10
Rubus occidentalis	Black raspberry		(a)
Rubus allegheniensis	Mountain blackberry		3
Rubus hispidus	Swamp dewberry	2	3
Rubus sp	Bramble	3	11
Dalibarda repens	Dalibarda	7	
Trifolium pratense	Red clover		3
Trifolium hybridum	Alsike clover		2
Trifolium sp	Clover		2
Oxalis acetosella	Common wood sorrel		48
Oxalis corniculata	Lady's sorrel		2
Viola papilionacea	Early blue violet	18	58
Viola conadensis	Canada violet	2	
Circaea intermedia	Enchanter's nightshade	8	24
Aralia nudicaulis	Wild sarsaparilla	7	
Osmorhiza longistylis	Sweet cicely		(a)
Cornus alternifolia	Alternate-leaved dogwood	2	
Monotropa uniflora	Indianpipe		2
Rhododendron nudiflorum	Purple azalea	7	3
Gaultheria procumbens	Wintergreen	2	2
Vaccinium pennsylvanicum	Low sweet blueberry	3	(a)
Trientalis americana	Starflower	3	16
Prunella vulgaris	Heal-all		3
Veronica officinalis	Common speedwell		5
Epifagus virginiana	Beechdrops	10	14
Plantago major	Common plantain		2
Galium triflorum	Sweet-scented bedstraw	2	3
Mitchella repens	Partridge berry	68	53
Viburnum alnifolium	Hobblebush	53	16
Viburnum acerifolium	Mapleleaf viburnum	2	2
Lobelia inflata	Indian tobacco		(a)
Sambucus canadensis	Common elder	10	10
Sambucus racemosa	Red-berried elder	3	2
Aster sp	Aster		2
Bidens discoidea	Small beggar-ticks		6
Hieracium aurantiacum	Devil's-paintbrush		3

[a] Species observed within the area but not encountered on plots.

The following species, most of which have a frequency of 10 per cent or more, are confined largely or wholly to the grazed area: *Aspidium noveboracense, Dicksonia punctilobula, Pilea pumila, Rumex obtusifolius, Polygonum hydropiper, Potentilla canadensis, Oxalis acetosella,* and *Circaea intermedia.* It is interesting to note the high frequency (48 per cent) of *O. acetosella* on the grazed area and its total absence in the hemlock-beech stand on the ungrazed area. Earlier work by the writer (*1*) at Heart's Content showed that this species was absent in the hemlock-beech association but was very common (frequency 40 per cent) in the hemlock consociation. On the grazed area, in the hemlock-beech association, the frequency of *O. acetosella* is even higher than in the ungrazed hemlock consociation. The writer can offer no explanation for this rather striking condition. Troup (*7*) states that in Europe the wood sorrel is one of the best indicators of favorable light and soil conditions in a forest.

Other species which, although having low frequency values, are considered characteristic of the grazed area are: *Anthoxanthum odoratum, Phleum pratense, Agrostis alba, Poa* sp., *Juncus tenuis, Phytolacca decandra, Stellaria media, Ranunculus acris, Podophyllum peltatum, Cardamine pennsylvanica, Potentilla recta, Trifolium pratense, Trifolium hybridum, Trifolium* sp., *Prunella vulgaris, Plantago major, Lobelia inflata, Bidens discoidea,* and *Hieracium aurantiacum.*

The number of herbaceous species on the grazed area is about 189 per cent of the number of species found on the ungrazed area. Soil conditions resulting from the presence of stock appear more favorable for the development of a rather rich herbaceous vegetation. The more favorable conditions appear to consist of increased light, better seedbed conditions due to the trampling of the animals, and reduced competition from shrubby and young tree growth. In addition, it is probable that stock may play an important rôle in seed dissemination. The seeds of such a plant as *Bidens discoidea* may be carried on the bodies of animals; seeds of *Phleum pratense, Trifolium pratense, T. hybridum* and others may be deposited with the dung. Some of the species appearing almost exclusively on the grazed area are considered to be nitrophilous, e. g., *Pilea pumila* and *Circaea intermedia.*

EFFECT OF GRAZING ON DENSITY OF VEGETATIVE COVER

Figure 3 shows graphically the effect of grazing animals on the vegetation as a whole. The degree of cover, in terms of percentage, for each of fiv esize classes in the grazed and ungrazed areas is indicated in solid black. Shrubby and herbaceous vegetation, as well as young tree growth, were considered in arriving at the degree of cover. In the smallest size class—the vegetative layer up to 1 foot in height—the degree of cover on the grazed area is almost twice that on the ungrazed area. Moreover, no other size class on the grazed area has so high a degree of cover. This high density is due chiefly to the increase in amount of herbaceous vegetation. The size classes above the general limit of herbaceous vegetation show a different condition. The degree of cover on the grazed area is only about 58 per cent of that on the ungrazed, in size class 2; 17 per cent in size class 3; 62 per cent in size class 4; and 102 per cent in size class 5. The effect of grazing thus appears to be maximum in the vegetative layer between 2.6 and 6 feet. In the next tallest layer, the com-

ponents of which probably have been longest exposed to browsing, grazing has reduced the density of cover from 38 per cent in the ungrazed area to 24 per cent in the grazed—nearly as great an absolute reduction as in size class 3. The practical uniformity, on both sides of the pasture fence, of cover density in the 15.1 to 25 foot size class indicates that the vegetation in this class was too tall when grazing began to have suffered appreciably from it.

EFFECT OF GRAZING ON THE SITE

No direct study of the effect of grazing on the site was carried out but observations were made during the course of the other field work. Trampling by the stock appears to have given the mineral surface soil on the grazed area a rather dense structure and compact consistency. From the standpoint of forest growth, this condition may be regarded as unfavorable. On the other hand, the presence of animals on the grazed area appears to have encouraged rapid decomposition of the organic débris. Over most of the area the humus

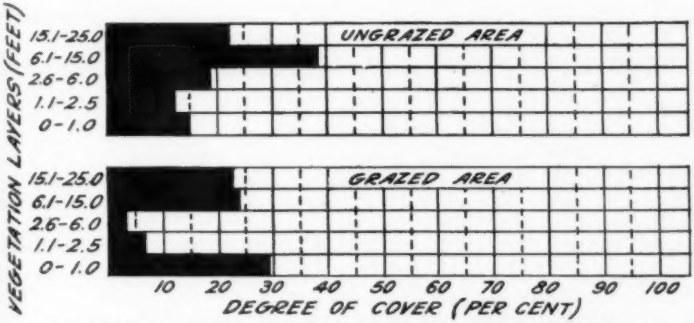

FIGURE 3.—Degree of cover in each of five size classes on the ungrazed and grazed areas

conditions appear considerably better than on the ungrazed area. Rather rapid nitrification is indicated by the fairly common occurrence of species which are generally regarded as nitrophilous. A study of the effect of grazing animals on the physical and chemical characteristics of the forest soil would be valuable.

SUMMARY

The effect of grazing on virgin forest vegetation was studied at Heart's Content, in northwestern Pennsylvania.

Direct injury to vegetation by grazing animals consists of browsing the tops, breakage by rubbing or trampling, root injuries due to trampling, and pulling small trees out of the soil. In the case of hemlock and beech a greater number of dead trees were recorded on the grazed than on the ungrazed area. However, comparative numbers of dead trees on the two areas are not considered as good an index of the severity of grazing injury as are comparative numbers of live trees.

Compared with the ungrazed area, the number of individuals of hemlock, red maple, and beech in size class 1 showed a large increase on the grazed area. In size classes 2, 3, 4, and 5, however, there was

a marked reduction in the number of individuals per acre on the grazed area. This reduction amounted to 61 per cent in the hemlock, 81 per cent in the red maple, and 85 per cent in the beech.

The basal area of trees 1 inch and over, diameter at breast height, in size classes 3, 4, and 5 on the grazed area was 7.84 square feet and on the ungrazed area was 19.26 square feet.

The total number of shrubby and herbaceous species was much greater on the grazed than on the ungrazed area. A number of species appeared exclusively on the grazed and others appeared exclusively on the ungrazed area.

The degree of cover on the grazed area in the five size classes recognized was in general notably lower than the degree of cover in the same size classes on the ungrazed area.

The surface soil on the grazed area was compacted due to the trampling by stock. Another result of the presence of the stock was the reduction in the amount of organic débris on the forest floor due to more rapid decomposition. A number of the plants on the grazed area are commonly recognized as nitrophilous.

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